Nurr1 Is Required for Maintenance of Maturing and Adult Midbrain Dopamine Neurons

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Transcription factors involved in the specification and differentiation of neurons often continue to be expressed in the adult brain, but remarkably little is known about their late functions. Nurr1, one such transcription factor, is essential for early differentiation of midbrain dopamine (mDA) neurons but continues to be expressed into adulthood. In Parkinson’s disease, Nurr1 expression is diminished and mutations in the Nurr1 gene have been identified in rare cases of disease; however, the significance of these observations remains unclear. Here, a mouse strain for conditional targeting of the Nurr1 gene was generated, and Nurr1 was ablated either at late stages of mDA neuron development by crossing with mice carrying Cre under control of the dopamine transporter locus or in the adult brain by transduction of adeno-associated virus Cre-encoding vectors. Nurr1 deficiency in maturing mDA neurons resulted in rapid loss of striatal DA, loss of mDA neuron markers, and neuron degeneration. In contrast, a more slowly progressing loss of striatal DA and mDA neuron markers was observed after ablation in the adult brain. As in Parkinson’s disease, neurons of the substantia nigra compacta were more vulnerable than cells in the ventral tegmental area when Nurr1 was ablated at late embryogenesis. The results show that developmental pathways play key roles for the maintenance of terminally differentiated neurons and suggest that disrupted function of Nurr1 and other developmental transcription factors may contribute to neurodegenerative disease.

Introduction

Adaptation to a changing environment requires plasticity in the adult CNS. However, to ensure that neurons are properly maintained, such plasticity must be balanced against mechanisms that counteract phenotypic instability. Studies of how neurons develop may help to unravel functions important for the stability of nerve cells as factors promoting their differentiation may also contribute to their maintenance. Indeed, many transcription factors identified for their critical roles during neuronal development continue to be expressed in the postnatal nervous system, raising the possibility that they contribute to the integrity of already differentiated neurons (Hendricks et al., 1999; Vult von Steyern et al., 1999; Kang et al., 2007; Alavian et al., 2008). However, the consequences of adult gene ablation of any of these factors have not yet been reported, and very little is known of their functions in differentiated neurons.

From a clinical perspective, it is of particular interest to identify factors that maintain stability of neurons that are affected in neurodegenerative disorders as loss of phenotype would likely cause or contribute to disease. Parkinson’s disease (PD) is characterized by progressive pathology of midbrain dopamine (mDA) neurons of substantia nigra pars compacta (SNc) and the ventral tegmental area (VTA), typically involving deposition of α-synuclein-rich cytoplasmic protein aggregates termed Lewy bodies. During development, early signaling events induce transcription factors that control the specification and differentiation of mDA neurons (Smidt and Burbach, 2007). Several of these factors, including Nurr1, Lmx1a, Lmx1b, Pitx3, FoxA2, and En1/2, continue to be expressed in the postnatal and adult brain (Zetterström et al., 1996; Smidt et al., 1997, 2000; Albéria et al., 2004; Simon et al., 2004; Kittappa et al., 2007). Nurr1, belonging to a family of ligand-independent nuclear receptors (Wang et al., 2004; Simon et al., 2004; Kittappa et al., 2007), is expressed in dopamine neurons of both the developing and adult midbrain, suggesting a role in the maintenance of mature dopaminergic neurons.

Nurr1, a member of the nuclear receptor superfamily, is a member of a family of ligand-independent nuclear receptors (Wang et al., 2004; Simon et al., 2004; Kittappa et al., 2007) that controls the specification and differentiation of mDA neurons in the developing midbrain (Hendricks et al., 1999; Vult von Steyern et al., 1999; Kang et al., 2007; Alavian et al., 2008). Nurr1 expression continues into adulthood and is required for the normal function of mDA neurons (Zetterström et al., 1996; Smidt et al., 1997, 2000; Albéria et al., 2004; Simon et al., 2004; Kittappa et al., 2007). Nurr1, belonging to a family of ligand-independent nuclear receptors (Wang et al., 2004; Simon et al., 2004; Kittappa et al., 2007), is expressed in dopamine neurons of both the developing and adult midbrain, suggesting a role in the maintenance of mature dopaminergic neurons.
For immunohistochemistry, sections were preincubated for 1 h in blocking solution containing either 10% normal goat serum or 5–10% bovine serum albumin, 0.25% Triton X-100, and 0.01% Na-azide in PBS. Primary antibodies diluted in blocking solution were applied overnight at 4°C. After washes with PBS, biotinylated- or fluorophore-conjugated secondary antibodies were followed by incubation with streptavidin–horseradish peroxidase complex (ABC elite kit, Vectorstain) for 1 h and subsequent exposure to diaminobenzidine (DAB kit; Vector Laboratories). Primary antibodies and dilution factors were as follows: rabbit anti-Nurr1 (1:100; M196; Santa Cruz Biotechnology), anti-Nurr1 (1:250; E20; Santa Cruz Biotechnology), rabbit anti-tyrosine hydroxylase (TH) (1:1500; Pel-Freeze), rat anti-DAT (1:2000; Millipore Bioscience Research Reagents), mouse anti-TH (1:200; Millipore Bioscience Research Reagents), rabbit anti-vesicular monoamine transporter (VMAT) (1:500; Millipore Bioscience Research Reagents), rabbit anti-t-DOPA decarboxylase (AADC) (1:500; Millipore Bioscience Research Reagents), rabbit anti-Cre (1:10,000; Covance Research Products), guinea pig anti-Lmx1b (1:1000; Anderson et al., 2006), and rabbit anti-Pitx3 (Smidt et al., 2004). In some cases (anti-AADC, anti-VMAT, and anti-Nurr1), the blocking steps were performed after antigen retrieval (Dako). Finally, expression was detected by secondary antibodies from Jackson ImmunoResearch. Section images were collected by confocal microscopy (Leica DMIRE2) and bright-field microscopy (Eclipse E1000K; Nikon). Cell counting was performed by counting all SNc DA neurons detected by immunohistochemistry (DAB) in a total of three sections per animal (every 12th tissue section) within the ventral midbrain. Animals taken at 4 months after vector injection in both wild-type wt and cNurr1AAVcre and cNurr1AAVCre animals. The mean of counted cells per animal was established from both the injected and non-injected sides in each animal, and the relative decrease was calculated as a percentage as described in Results.

Measurements of tissue content for dopamine, serotonin, and their metabolites. In supplemental Tables 1–3 (available at www.jneurosci.org as supplemental material), tissues were collected from P1, P7, P14, and adult (P48) pDATcre and cNurr1AAVcre mice. One- to 14-d-old mice were killed by decapitation, and the brains were collected. Brain tissues were rapidly removed, chilled in saline (4°C), dissected, frozen on dry ice, and stored at -80°C until use. To process tissues for HPLC and electrochemical detection of monoamines and metabolites, samples were homogenized by sonication in 50 vol or in 30 µl of 0.1 M perchloric acid, followed by centrifugation. Endogenous levels of noradrenaline, DA, 3,4-dihydroxyphenylacetic acid, homovanillic acid (HVA), serotonin (5-HT), and 5-hydroxyindoleacetic acid were determined in the supernatants. A reverse column (BAS, C-18, 100.0 × 3.2 mm, 3 µm particle diameter) was used for separation. The mobile phase consisted of 0.05 M sodium phosphate/0.03 M citric acid buffer containing 0.1 mM EDTA and was adjusted with various amounts of methanol and sodium-t-octane sulfonylic acid. The flow rate was 0.3 ml/min. Monoamines and metabolites were detected using a glassy-carbon electrode detector, which is set at +0.7 V versus an Ag/AgCl reference electrode. Resultant peaks were measured and compared with repeated control samples containing fixed mixed amounts of compounds of interest.
line crossing was counted when the mouse moved its whole body from one square to another.

Stepping test. Forelimb akinesia was monitored in a modified version of the stepping test, as described previously for rats (Schallert et al., 1992; Kirik et al., 1998). The test was performed three times daily over 3 consecutive days. In this test, the mouse was held firmly by the experimenter with both hindlimbs and one forelimb immobilized, and the mouse was passively moved with the free limb contacting a table surface. The number of adjusting steps, performed by the free forelimb when moved in the forehand and backhand directions, over a distance of 30 cm, was recorded. Results are presented as data collected on the third testing day.

Results

Selective Nurr1 ablation in late developing mDA neurons

A mouse strain containing a Nurr1 allele for conditional gene ablation was generated by insertion of two loxP sequences in the second and third introns so that the coding sequence, including the first coding exon 3, is excised by Cre-mediated recombination (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). To analyze the consequences of Nurr1 ablation at late stages of mDA neuron development, we crossed floxed Nurr1 mice with mice carrying Cre inserted in the locus of the DAT gene (Ekstrand et al., 2007). Crosses generated Nurr1 mice that were homozygous for the conditional targeted Nurr1 allele and heterozygous for the DAT– Cre allele (Nurr1L2/L2; DATCre/wt, hereafter referred to as cNurr1DATCre mice). Littermates of genotype Nurr1L2/L2; DATwt/wt or Nurr1L2/wt; DATCre/wt were used as controls. Although we cannot exclude that a small number of cells escape Nurr1 gene deletion, immunohistochemistry using an antibody against Nurr1 showed that DAT– Cre-mediated Nurr1 ablation resulted in the expected delayed loss of Nurr1 expression in mDA neurons beginning from approximately E13.5 and becomes essentially complete at E15.5 (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). At this stage of normal development, cells express pan-neuronal properties as well as many mDA neuron markers, and axons are growing toward the developing striatum (Smidt and Burbach, 2007).

cNurr1DATCre mice were born at the expected Mendelian frequency of ~25% (of a total n = 159); however, cNurr1DATCre mice were less active than controls and did not survive beyond 3 weeks after birth. If litters were allowed to remain with their mothers after weaning, perinatal death was avoided in ~50% of cNurr1DATCre pups. These surviving mice were, however, ~40% smaller than controls at the age of 2 months (supplemental Fig. 3, available at www.jneurosci.org as supplemental material). Although no significant change in spontaneous light-phase locomotor activity could be observed in adult cNurr1DATCre mice, rearing was dramatically decreased (supplemental Fig. 3, avail-

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**Figure 1.** TH is progressively lost in both the ventral midbrain and striatum of cNurr1DATCre mice. **A–T,** Confocal microscopy showing TH immunohistochemistry in control (ctrl) and cNurr1DATCre as indicated. **A–J,** Sections were analyzed at the levels of ventral midbrain (as indicated in **A** and **F**) and in the striatum (as indicated in **K** and **P**). TH immunofluorescence was analyzed at both embryonal and postnatal stages as indicated. Results demonstrate a progressive loss of TH immunoreactivity in the ventral midbrain. Note that TH immunoreactivity was more drastically downregulated at more lateral regions compared with the prospective medial VTA. **K–T,** TH immunoreactivity in the striatum. In cNurr1DATCre mice, TH was lost in the CPu and diminished in the NAc. Scale bars, 250 μm.
able at www.jneurosci.org as supplemental material). l-DOPA treatment of mutant mice did not improve viability and did not induce any weight gain. Instead, cNurr1DATCre mice display a pronounced and severe hypersensitivity to l-DOPA treatment characterized by an acute phase of hyperactivity and repetitive behaviors (including repetitive gnawing, excessive grooming, and self-injury) in all tested mutant (n = 9) but not in any wild-type controls (n = 7) (see Materials and Methods). These behaviors resemble those that have been observed in neonatal 6-hydroxydopamin lesioned rats treated with l-DOPA (Breese et al., 2005). In conclusion, late embryonic mDA neuron-selective Nurr1 ablation is associated with decreased weight, rearing, and viability, and mice show an altered response to l-DOPA.

Reduced levels of TH and DA in brains of cNurr1DATCre mice
The observed abnormalities are consistent with a dopaminergic deficiency. To analyze the possible cellular basis for the phenotype, brain sections from controls and cNurr1DATCre mice were analyzed by immunohistochemistry using an antibody against TH (Fig. 1). A progressive loss of TH immunostaining in SNc was observed in the cNurr1DATCre mice (Fig. 1A–J). TH levels were significantly decreased already at E15.5, soon after Nurr1 is lost, and decreased further until adulthood when only scattered TH-positive neurons could be detected. TH was diminished also within the VTA at later stages, but a significant number of cells remained even in adult sections (Fig. 1A–J). These cells were counted in four non-consecutive sections for each analyzed brain. In adult control VTA, a mean of 74.5 ± 7.1 cells per section were counted in cNurr1DATCre mice (n = 4) and 499.3 ± 5.2 cells in controls (n = 3) (Student’s t test, 4.4 × 10−7). TH immunostaining within the caudatus putamen (CPu) was completely lost (Fig. 1A–T). However, weak immunoreactivity remained in nucleus accumbens (NAc) innervated preferentially by VTA neurons (Fig. 1A–T). We also noted the appearance of ectopic TH-positive cell bodies within the striatal parenchyma in cNurr1DATCre mice (supplemental material, available at www.jneurosci.org as supplemental material). These cells were more frequent in regions in which striatal TH had been most severely depleted as a consequence of Nurr1 ablation and resemble TH-positive neurons appearing in rodent and primate DA-depletion models (Huot and Parent, 2007). Decreased levels of TH immunostaining were paralleled by decreased DA levels, as measured by HPLC (supplemental Tables 1–3, available at www.jneurosci.org as supplemental material). Striatal DA was drastically reduced to 14% of controls at P1 in cNurr1DATCre mice and was almost completely lost by P60. An increased ratio of HVA to DA at P14 indicated increased turnover of DA in remaining cells at this stage (supplemental Table 2, available at www.jneurosci.org as supplemental material). DA was more severely decreased in CPu compared with NAc (supplemental Table 3, available at www.jneurosci.org as supplemental material). In contrast, 5-HT was significantly increased in both CPu and NAc, consistent with previous findings showing increased serotonergic innervation after striatal DA depletion (Snyder et al., 1986). Thus, a severe neurotransmitter deficiency of the mesostriatal DA system is apparent in cNurr1DATCre mice. Together, measurements of TH immunoreactivity and DA levels demonstrate that Nurr1 is critically required for maintaining TH expression and DA synthesis from late stages of mDA neuron differentiation.

Cellular deficiency within the ventral midbrain of cNurr1DAT Cre mice
To investigate whether the phenotype is a consequence of a more limited disruption of DA synthesis or a more severe cellular deficiency, a number of additional mDA neuron markers were analyzed. All analyzed mDA neuron markers were diminished or absent within SNc in cNurr1DATCre mice already at E15.5 (Fig. 2). DAT was completely lost at E15.5 and therefore, consistent with previous data (Sacchetti et al., 1999), stands out as being a likely direct target of Nurr1 (Fig. 2A–D). Additional control experiments showed that DAT and other markers, including TH and

![Figure 2](https://www.jneurosci.org/supplemental_table/Figure_2.png)
Nurr1, were not visibly decreased in mice heterozygous for the DAT–Cre allele (supplemental Fig. 5, available at www.jneurosci.org as supplemental material) (data not shown). In contrast to DAT, AADC, VMAT2, Pitx3, or Lmx1b were not reduced within the most medial ventral midbrain at this early stage and, with the exception of DAT, markers were not completely downregulated at P1 (Fig. 2E–T). The progressive loss of markers indicates a severe loss of phenotype within the SNc, whereas cells within the VTA appear more resilient. Importantly, most TH-positive cells within the VTA have lost any detectable expression of DAT, indicating that these cells have not escaped Nurr1 gene targeting (supplemental Fig. 6, available at www.jneurosci.org as supplemental material).

To further assess the extent of a cellular deficiency, striatal target innervation was analyzed by Fluorogold retrograde tracing after injection into the striatum of live 8- to 9-week-old controls and cNurr1DATCre mice. Fluorogold was transported into SNc cell bodies of control mice; however, fluorescence was entirely undetected within the SNc of Fluorogold-injected cNurr1DATCre mice (Fig. 3, compare D, E with G, H). In addition, characteristic large and densely packed TH-immunoreactive mDA neurons within the SNc were virtually absent in control mice (Fig. 3, compare I–L). Striatal site of Fluorogold injection is marked by asterisk in A, B, and G. Scale bars: A, B, D, E, G, H, 1 μm; F, I, 1200 μm.

**Figure 3.** Cell bodies and striatal innervation are lost in cNurr1DATCre mice as determined by Fluorogold (FG) retrograde tracing of fibers extending from cell bodies in SNc to the striatum. A–C, After Fluorogold injection, injected into the left striatum in either adult (1.5 months old) control (Ctrl) or cNurr1DATCre mice as indicated in C, mice were killed after 4 d and analyzed for TH immunofluorescence in the striatum (A) or ventral midbrain (B). D–I, Analysis for Fluorogold (FG) or by Nissl staining. Strong Fluorogold staining in both striatum (D) and in the ventral midbrain (E) was consistently seen in all control (Ctrl) animals (n = 7). In contrast, Fluorogold fluorescence was only detected in the striatum (G) in cNurr1DATCre mice (n = 5), indicating that fibers from the SNc (H) had been lost in these animals. Moreover, large, densely packed cell bodies are only visualized by Nissl staining in the ventral midbrain of control animals (F) but are completely absent from cNurr1DATCre mice (J). Striatal site of Fluorogold injection is marked by asterisk in A, B, and G. Scale bars: A, B, D, E, G, H, 1 μm; F, I, 1200 μm.

**Figure 4.** TH expression in both the ventral midbrain and striatum is progressively lost in the injected, but not non-injected, side of cNurr1DATCre mice. A–H, Sections from 0.5, 1.5, and 4 month (mo; as indicated) old AAV–Cre injected controls (wtAAVCre) or cNurr1AAVCre mice were used for analyses by nonfluorescent DAB TH immunostaining in the ventral midbrain. The analyzed region within the ventral midbrain is schematically illustrated in A and E. The site of injection, marked by an asterisk in B–D and F–H, was verified in all animals by high-power magnification microscopy and was identified as a small area of injection-induced necrosis. Results show that TH immunostaining is not drastically altered at 0.5 months but is progressively decreased at 1.5 and 4 months in the injected SNc and VTA. I–L, DAB TH staining at the level of striatum. Analyzed regions are indicated in I. TH staining is progressively decreased at 1.5 and 4 months in the side that is ipsilateral to the side of AAV–Cre injection in cNurr1AAVCre mice (J–L). OT, Olfactory tubercle. Scale bars: A–H, 600 μm; I–L, 1 mm.

Adeno-associated virus–Cre-mediated Nurr1 ablation in adult mice

In cNurr1DATCre mice, Nurr1 is ablated well before full mDA neuron maturity and before targets in the striatum have become innervated; thus, it remained possible that the phenotype is a consequence of a developmental dysfunction. Therefore, we proceeded to inactivate Nurr1 specifically in ventral midbrain of adult mice, using an adeno-associated virus (AAV)–Cre vector driven by the neuron-specific synapsin promoter. AAV–Cre was administered by unilateral stereotaxic microinjection above the right SNc. Cre immunohistochemistry and β-galactosidase expression was analyzed after intranigral AAV–Cre injection into reporter mice in which the ROSA26 locus is targeted with a LacZ reporter gene (Soriano, 1999). Results show widespread Cre expression around the site of injection, spreading into both SNc and VTA, and robust recombination of the LacZ reporter construct (supplemental Fig. 7, available at www.jneurosci.org as supple-
AAV–Cre was unilaterally injected above the SNc of adult conditional gene-targeted mice (cNurr1AAVCre) or into wild-type control mice (wtAAVCre). In addition, a vector encoding the green fluorescent protein (GFP) driven by the synapsin promoter (AAV–GFP) was injected in mice homozygous for the floxed Nurr1 allele (cNurr1AAV–GFP Cre) to ensure that the floxed animals are not more sensitive to nonspecific toxicity induced by AAV transduction. Histological analyses were performed from animals killed at 0.5, 1.5, and 4 months after injection.

Reduction of TH and DA in adult Nurr1-ablated mice

TH immunohistochemistry at the level of the ventral midbrain was analyzed to assess the consequences of adult Nurr1 ablation. Within SNc, TH immunoreactivity was unaffected at 0.5 months but was progressively reduced at 1.5 and 4 months in the injected SNc in cNurr1AAVCre mice (Fig. 4 E–H). In contrast, TH immunoreactivity was unaffected in SNc of control wtAAVCre and cNurr1AAV–GFP Cre mice (Fig. 4 A–D) (data not shown). TH was also reduced in the VTA at 1.5 and 4 months; however, at 4 months, the reduction in VTA was less dramatic compared with SNc (Fig. 4 E–H).

Decreased striatal TH immunoreactivity paralleled the reduction in the ventral midbrain. Thus, although no signs of degenerating striatal TH-stained fibers (swollen axons or dystrophic neurites) were detected, striatal sections ipsilateral to the side of AAV–Cre injection showed clearly reduced TH in cNurr1AAVCre mice but not in controls (wtAAVCre or cNurr1AAV–GFP Cre) (Fig. 4 I–L) (supplemental Fig. 8, available at www.jneurosci.org as supplemental material). Diminished TH immunoreactivity was observed in regions innervated by both SNc and VTA (CPu and NAc, respectively), consistent with the reduced TH immunoreactivity in both SNc and VTA mDA neuron cell bodies. Measurement of DA and metabolites by HPLC from dissected tissue at 4 months confirmed this picture as a significant reduction in DA and DA metabolites noted both within the dorsolateral striatum and in areas mostly innervated by the VTA (cortex and ventromedial striatum) (supplemental Table 4, available at www.jneurosci.org as supplemental material). Thus, TH, DA, and DA metabolites are clearly reduced as a result of adult Nurr1 ablation.

Loss of mDA neuron characteristics in adult Nurr1-ablated mice

To further analyze the fate of Nurr1-ablated neurons, cells were counted within the SNc and VTA in cNurr1AAVCre and wtAAVCre mice. Within SNc, the number of TH-positive cells was significantly decreased at 4 months (58.1 ± 8.3 and 95.4 ± 6.3% in the injected vs non-injected sides of cNurr1AAVCre and wtAAVCre mice, respectively; p = 0.0053). In contrast, the numbers of TH-positive cells was not significantly reduced within the VTA (104.1 ± 4.7 and 101.6 ± 10.5% in the injected versus non-injected sides of cNurr1AAVCre and wtAAVCre mice, respectively). Also, the numbers of TH-positive cells were not significantly changed in cNurr1AAVCre mice at 1.5 months (data not shown).

To assess the integrity of neurons, cellular analysis was extended by analyzing Cre-immunolabeled sections that were superimposed on adjacent TH-labeled sections (Fig. 5). Notably, in cNurr1AAVCre mice, Cre expression was clearly detected within the area of SNc at both 0.5 and 1.5 months but was lost at 4 months in the region in which mDA neurons should normally be localized (Fig. 5, compare E–G with B–D). Cre expression is driven by a general neuronal promoter (synapsin), suggesting that loss of Nurr1 may eventually affect some pan-neuronal properties at 4 months after ablation.

Confocal microscopy confirmed the loss of TH at 1.5 and 4 months after Nurr1 ablation and the loss of Cre at 4 months (Fig. 6A–D). At 1.5 months, DAT expression was weak but cell bodies were readily identified (Fig. 6E, F and inset in F). DAT staining remained also at 4 months, but, at this stage, high-power magnification indicated that some of the staining appeared confined to fibers and/or dystrophic cells (Fig. 6G, H and inset in H). Nonetheless, at 4 months, most cells with decreased TH stained positive for AADC, showing that not all mDA neuron characteristics were affected (Fig. 7A–F). Moreover, VMAT2 is yet another marker that was severely decreased in cNurr1AAVCre, but remaining weakly stained cells were positive for the general neuronal marker Hu (Fig. 7G–L). The observed changes were not correlated to increased number of apoptotic cells because increased activated Caspase 3 could not be detected (data not shown). Also, we found no evidence for nigral inflammation or α-synucleinopathy because activated microglia and α-synuclein-rich inclusions were not detected at any stage after Nurr1 ablation in cNurr1DATCre mice (data not shown). Finally, TH and DAT expression in VTA was also affected, without any apparent loss of the neuronal marker Hu or any signs of dystrophic cells (supplemental Fig. 9, available at www.jneurosci.org as supplemental material) (data not shown). Thus, Nurr1 ablation results in a
progressive dysfunction characterized by a partial loss of the mDA neuron phenotype. Although we see few signs of neuronal degeneration, we cannot exclude a limited cell loss.

To assess whether the observed dysfunction was paralleled by an altered motor behavior, cNurr1AAVCre mice were subjected to a stepping test at 3 and 4 months (Schallert et al., 1992; Kirik et al., 1998). Performance of the left forelimb (i.e., the limb contralateral to the vector injection) was impaired at both time points (Fig. 8). Additional behavioral testing, including amphetamine-induced rotations and a corridor test, indicated that individual mutant animals appeared affected; however, the Nurr1-ablated group did not show alterations that were statistically significant (supplemental Fig. 10, available at www.jneurosci.org as supplemental material). Our results demonstrate progressive mDA neuron dysfunction, leading to a more severe deficiency at 3–4 months after Nurr1 ablation.

Discussion
This study provides definitive evidence that Nurr1 is not only critical for early differentiation but also for the maintenance of functional mDA neurons. Conditional gene targeting at late embryogenesis, when characteristic features of mDA neurons are already apparent, results in a rapid and close to complete mDA neuron loss. Only few TH-positive cells remain within the VTA also in the absence of Nurr1. Removal of Nurr1 leads to a severe dysfunction also in adult mDA neurons. It should be noted that reduction of striatal DA and the behavioral effects after adult ablation most likely underestimate the importance of Nurr1 in the adult brain because AAV injection only transduced a proportion of all mDA neurons in the injected side of treated animals. Thus, these data emphasize the importance of studying developmental mechanisms for elucidating neuron maintenance mechanisms. An analogous example is provided by the glial cell line-derived neurotrophic factor (GDNF). GDNF is known to promote neuronal survival under development, but only recently has conditional gene targeting enabled studies that interrogate the role of GDNF and other factors signaling via RET for maintenance of midbrain dopamine neurons in the adult brain (Oo et al., 2003; Jain et al., 2006; Kramer et al., 2007; Pascual et al., 2008).

Data presented here have implications for our understanding of how mature differentiated cell types are maintained. Previous studies have indicated that the differentiated state is not irreversible because even mature specialized cells, including for example, olfactory neurons and mature T- and B-cells, can be reprogrammed into undifferentiated pluripotent cells by either somatic cell nuclear transfer or using the recently developed methodology for the generation of induced pluripotent stem cells (Takahashi and Yamanaka, 2006; Gurdon and Melton, 2008). Nevertheless, under normal nonmanipulated conditions in vivo, differentiated cells are remarkably stable, indicating the importance of mechanisms that maintain cells in their appropriate differentiated state. Gene targeting in non-neural cell types has revealed how transcription factors functioning in development can be important for the maintenance of terminally differentiated cell types, e.g., Pax5 in B-lymphocytes and Prox1 in lymphatic endothelial cells (Cobaleda et al., 2007; Johnson et al., 2008). In CNS, transcription factors identified for their key roles in early neuron development often continue to be expressed in the adult brain and may therefore guard against loss of phenotype or drift into alternative states (Smidt et al., 1997, 2000; Zetterström et al., 1997; Hendricks et al., 1999; Vult von Steyern et al., 1999; Albéri et al., 2004; Simon et al., 2004; Kang et al., 2007; Kittappa et al., 2007; Smidt and Burbach, 2007; Alavian et al.,

Figure 6. Decreased expression of DAT and signs of dystrophic cells in cNurr1AAVCre mice. A–H, Confocal analysis of SNc in wtAAVCre and cNurr1AAVCre mice at 1.5 and 4 months, as indicated. Confocal images show double staining of Cre (red) and TH (green; A–D) and staining for DAT (green; E–H). Micrographs show that there is a loss of TH and DAT and a progressive loss of synapsin-driven Cre at 4 months. At 4 months, DAT staining appears fragmented and stains scattered fibers, whereas very few intact cell profiles (marked with arrowheads in F and H) can be identified in cNurr1AAVCre mice (compare insets in F, H). Scale bar, 200 μm.
However, remarkably little is known of how these factors function at late stages of development or in the adult. Although examples of adult mDA neuron loss has been reported in mice haploinsufficient for transcription factor genes such as Engrailed and FoxA2, it remains possible that defects originate during embryonic development (Alberi et al., 2004; Zhao et al., 2006; Kittappa et al., 2007; Sonnier et al., 2007). Importantly, FoxA2 and Engrailed are critical for the establishment of the floor plate and for early midbrain/hindbrain development, respectively, and they are directly and indirectly affecting many cell fates along the entire neuraxis. Thus, haploinsufficiency may cause embryonal deficiencies that do not become manifest until adult stages, a possibility that emphasizes the importance of temporally controlled conditional gene targeting to rigorously test how transcription factors function in terminally differentiated neurons.

We do not yet understand why Nurr1 is required in already differentiated mDA neurons. However, data presented here provide compelling evidence for the existence of “terminal selector genes” in mammalian CNS development. Such genes, defined from studies of Caenorhabditis elegans neuronal development, are continuously expressed throughout the life of neurons and are essential for both the establishment and maintenance of distinct neuronal phenotypes (Hobert, 2008). Thus, Nurr1, which probably regulates typical mDA neuron markers such as TH, DAT, AADC, and VMAT2 (Sakurada et al., 1999; Sacchetti et al., 2001; Hermanson et al., 2003; Kim et al., 2003), is likely required for both early differentiation and maintenance by regulating genes that distinguish mDA neurons from other neuron types. Presumably, such regulation is critical throughout the life of mDA neurons and would depend on additional components, such as Pitx3, in a core transcription factor network (Jacobs et al., 2009).

How may dysregulated Nurr1 activity contribute to PD? Studies in PD patients have shown that, in early stages of the disease, SNC cell bodies are relatively spared compared with the loss of DA in the putamen (Fearnley and Lees, 1991) and that a significant fraction of the surviving, pigmented, DA somata in the SNC have much reduced expression of the TH enzyme (Hirsch et al., 1988; Chu et al., 2006). This suggests that, during early stages of disease, nigral DA neurons may survive in a dysfunctional state characterized by a downregulated neurotransmitter machinery. An interesting possibility supported by our data is that reduced expression of Nurr1 contributes to such symptoms. Indeed, Nurr1 is severely reduced in neurons with signs of pathology in PD brain tissue, and reduced Nurr1 expression in patients’ peripheral blood lymphocytes indicates that diminished Nurr1 activity may be a systemic feature of disease (Chu et al., 2006; Le et al., 2008). Although such correlations do not determine whether reduced Nurr1 expression is a cause or a consequence of disease, progressive cell dysfunction in Nurr1-ablated mice provides a clear indication that diminished Nurr1 expression in PD should have deleterious consequences for patients. This view is supported by the identification of Nurr1 gene variants that have been associated with rare cases of familial and sporadic PD (Xu et al., 2002; Le et al., 2003; Zheng et al., 2003; Jankovic et al., 2005; Grimes et al., 2006; Jacobsen et al., 2008). Although other studies have failed to identify such mutations and indicated that Nurr1...
Forelimb akinesia in the stepping test. The performance of the left paw (contralateral to the vector injection) was significantly impaired in the fl/fl mice (n = 16) but not the wild-type mice (n = 13). Although the impairment was significant at both time points, 3 and 4 months after vector injection, their performance got significantly worse over time (**p < 0.01, Student’s paired t test); 15 of the 16 mice in the fl/fl group showed a decline in their stepping scores between the two tests, and at 4 months, 14 of the fl/fl mice had scores below 5 compared with 6 in the 3 month test. Scores give the means of steps recorded in the forehand and backhand direction for each paw (see Materials and Methods). *p < 0.001, Student’s paired t test.

Figure 8.

In conclusion, loss of Nurr1 at stages when characteristic features of mDA neurons are already apparent in the developing embryo or in fully differentiated adult neurons results in loss of mDA neuron-specific gene expression and neuron degeneration. These findings highlight the importance of developmental mechanisms also in the adult brain and clearly indicate that they may be critical for the understanding of cell maintenance and neurodegeneration. How Nurr1 and other transcription factors operate in adult neurons to control and prevent loss or drift in phenotype remains a challenge for future studies.

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