Dopamine Neuron Agenesis in Nurr1-Deficient Mice

Rolf H. Zetterström, Ludmila Solomin, Lottie Jansson, Barry J. Hoffer, Lars Olson, Thomas Perlmann*

Dopamine neurons of the substantia nigra and ventral tegmental area regulate movement and affective behavior and degenerate in Parkinson's disease. The orphan nuclear receptor Nurr1 was shown to be expressed in developing dopamine neurons before the appearance of known phenotypic markers for these cells. Mice lacking Nurr1 failed to generate midbrain dopaminergic neurons, were hypoactive, and died soon after birth. Nurr1 expression continued into adulthood, and brains of heterozygous animals, otherwise apparently healthy, contained reduced dopamine levels. These results suggest that putative Nurr1 ligands may be useful for treatment of Parkinson's disease and other disorders of midbrain dopamine circuitry.

Dopamine (DA) midbrain neurons function in the control of movement and their loss is the cause of Parkinson's disease. Mechanisms underlying dopaminergic development are largely unknown. Whereas general induction of ventral cell fate requires the secreted factor sonic hedgehog (*1*), additional factors must contribute to differentiation of specific cell types such as DA neurons. Glial cell line–derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor have been demonstrated to promote survival of DA cells (*2*), but the absence of these factors does not affect prenatal DA neuron development (*3*). In contrast, Nurr1, an orphan member of the steroid/thyroid hormone receptor family (*4*), which is expressed in DA cells (*5*), was found to be absolutely required for development of midbrain DA neurons.

Nurr1 genomic DNA was used to construct a targeting vector (Fig. 1A) in which a neomycin phosphotransferase gene replaced the NH_2-terminal transactivation and DNA-binding domains of Nurr1. From cross-breeding of heterozygous Nurr1 mutant mice, we obtained homozygous animals ($Nurr1^{-/-}$; Fig. 1, B and C) born at the expected frequency of 25% without apparent abnormalities. Homozygous mice could be distinguished after birth by hypoactivity and the lack of milk in their stomachs and died within the first 2 days after birth. Histology of mutants did not reveal any gross morphological abnormalities of brain, adrenals, thymus, lungs, heart, liver, kidneys, or intestine. Peripheral nerves, spinal cord, and dorsal root ganglia appeared normal in $Nurr1^{-/-}$ mice as judged by laminin, protein gene product 9.5, neurofilament, and calcitonin gene-related peptide immunohistochemistry (*6*).

Nurr1 mRNA is expressed in substantia nigra pars compacta (SNC) and the ventral tegmental area (VTA) (*5*). Expression appears at embryonic day 10.5 (E10.5) in the ventral aspect of the mesencephalic flexure where DA neurons later develop but before known phenotypic markers for DA neurons such as tyrosine hydroxylase (TH) can be detected (Fig. 2). TH immunoreactivity (IR) (*6*) was absent in $Nurr1^{-/-}$ mice in the ventral midbrain where TH-positive cell groups (defined as A8 through A10) (*7*) are normally present (Fig. 2, E and H). TH-IR was present in newborn $Nurr1^{-/-}$ mice in other areas known to express TH (Fig. 2, F, G, I, and J). TH-IR, normally appearing in the developing midbrain at approximately E11.5 in mouse (*8*), was absent throughout all prenatal stages (from E11.5 to newborn; Fig. 2, K through N). Therefore, the data suggest that Nurr1 is critical for midbrain dopaminergic cell differentiation.

Although Nurr1 may directly activate transcription of the TH gene, this seems unlikely as most TH-positive cell groups do not express Nurr1 mRNA (*5*), and these other cell groups were TH immunoreactive in $Nurr1^{-/-}$ mice (Fig. 2). Instead, Nurr1 appears to determine the specific dopaminergic cell fate. Analysis of cresyl violet–stained sections (*6*) revealed a disorganized distribution of cells in the ventral midbrain of $Nurr1^{-/-}$ mice, suggesting that TH-expressing neurons fail to differentiate. In situ hybridization showed that Nurr1 mRNA was expressed in SNC and VTA in wild-type animals, whereas no hybridization signal was detected in $Nurr1^{-/-}$ mice (Fig. 3, A and B). Neither TH-IR nor TH mRNA (Fig. 3, C and D) was found in mutant midbrains. Additional DA neuron markers, including aldehyde dehydrogenase 2 (ADH2) (*9*), mRNA for the GDNF signal transducing receptor c-Ret (Fig. 3, E through H) (*10*), and the DA D_2 receptor were also absent in SNC and VTA in $Nurr1^{-/-}$ mice.

The cause of death in $Nurr1^{-/-}$ mice is not clear although it appears to involve inability to suckle. Mutant mice display movement disturbances including severe difficulties in turning when placed on their backs and abnormal flexion-extension movements of limbs. The cause of death does not seem to involve competition with normal littermates, because litter restriction did not influence survival of the mutant pups. In mice where the TH gene was disrupted in dopaminergic cells, homozygotes survived 2 weeks after birth (*11*). Such delayed death is consistent with the postnatal course of development of striatal DA innervation (*12*) and suggests that perinatal lack of midbrain DA is not immediately lethal. However, absence of DA neurons, as is the case in the $Nurr1^{-/-}$ mice, rather than lack of transmitter, is accompanied by additional deficits in neuropeptides and neuronal circuitry (*13*). Alternatively, Nurr1

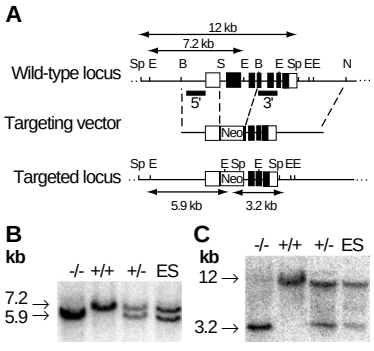

Fig. 1. Targeting the mouse Nurr1 locus (*21*). (**A**) Schematic representation of the Nurr1 wild-type locus, targeting vector, and targeted locus. Untranslated and translated exons are indicated by open and filled boxes, respectively. In the targeted locus, a thymidine kinase promoter–driven neomycin resistance gene (neo) replaces exons 2, 3, and part of exon 4. The expected fragment lengths in diagnostic Southern blot experiments are indicated by arrows for the wild-type (above) or targeted (below) locus, respectively. Probes located 5′ or 3′ (black bars) of the neo cassette were used. Restriction endonucleases are Sph I (Sp), Eco RI (E), Bam HI (B), Sal I (S), and Not I (N). For convenience, only those Sph I sites relevant for results in Southern blots are indicated. (**B**) Southern blot analysis was performed with the 5′ probe of Eco RI–cut DNA derived from mice originating from one of the injected ES cell lines. Lanes are homozygote (−/−), wild-type (+/+), and heterozygote (+/−) DNA. (**C**) Shown is a Southern blot of Sph I–cut DNA using the 3′ probe.

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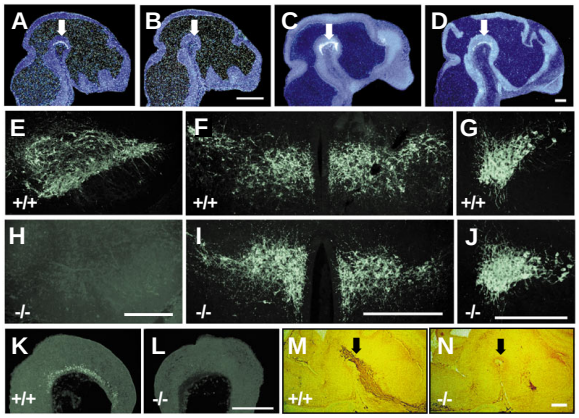

Fig. 2. In situ hybridization (*22*) of Nurr1 and TH mRNA (**A** through **D**), and TH immunohistochemistry (**E** through **N**) (*6*). Nurr1 mRNA was found in the ventral aspect of E10.5 mouse embryo midbrain (A) (indicated by arrow), at which time no TH mRNA could be detected within this region (B). At E11.5, when Nurr1 mRNA was strongly expressed in the mesencephalic flexure (C) (*14*), TH mRNA was also detected (D). (E through N) TH-IR in wild-type (+/+) and Nurr1 (−/−) mutants are compared. Coronal (E through J) and sagittal (K through N) sections of different stages are shown. In SNC (A9), TH-IR is strong in newborn wild-type mice (E) but completely absent in Nurr1 mutants (H). Distribution of TH-IR in the dopaminergic cell groups A11/A13 (*7*) of diencephalon (F and I) and the pontine locus coeruleus [A6 (G and J)], showing nuclei where no differences in TH-IR can be seen between wild-type and Nurr1$^{-/-}$ newborn brains. TH-IR was completely absent in ventral mesencephalon of Nurr1 mutants both at E11.5 (K and L) and E15.5 (M and N). At E15.5, TH-positive fibers, detected by peroxidase anti-peroxidase IR (*6*), extended rostrally from the midbrain in wild-type mice (M). A diencephalic TH-positive cell group was detected in the Nurr1$^{-/-}$ brain (N). Note the absence of TH in ventral mesencephalon (N). Arrows indicate the mesencephalic flexures (M and N). Scale bars, 300 μm.

could have vital functions at other sites of expression (*5*, *14*).

Striatum develops many organotypic features in genetically engineered DA-deficient mice (*11*) after perinatal ablation of dopaminergic neurons (*15*) or when grafted into 6-hydroxy-DA–lesioned adult rat brains (*16*). Our data support these findings. We found that mRNAs encoding enkephalin, substance P (Fig. 4, A and B), and choline acetyltransferase were normally distributed in striatum of Nurr1 mutant brains, although substance P mRNA levels appeared somewhat reduced, in agreement with previous studies of mice deficient in the DA D_1 receptor, as well as DA (*11*, *17*).

High-pressure liquid chromatography (HPLC) was used to measure levels of DA and the DA metabolite DOPAC in pieces of striatum and ventral midbrain (*18*). DA was absent in Nurr1$^{-/-}$ animals (Fig. 4C). Newborn heterozygotes showed reduced levels of DA (Fig. 4C) and DOPAC, indicating that nigrostriatal DA levels are affected by Nurr1 gene dosage. Adult heterozygotes also manifested reduced striatal DA levels ($P < 0.01$) but no apparent histological or behavioral abnormalities. This indicates that Nurr1 helps maintain the differentiated DA neuron phenotype in mature animals and suggests that DA levels could be regulated by Nurr1 ligands.

Understanding DA neuron development will involve elucidating the relationship of Nurr1 to sonic hedgehog signaling and to other regulatory components involved in midbrain patterning and cell fate specification (*1*, *19*). Nurr1 can promote signaling through heterodimerization with the 9-*cis* retinoic acid receptor, RXR (*20*). The enzyme ADH2, used here as a DA neuron marker, may have importance for retinoid synthesis (*9*). Thus, retinoids may activate RXR-Nurr1 heterodimers in developing DA neurons. Further insight into how retinoids, as well as a putative ligand for Nurr1, may influence the development and survival of DA neurons may provide opportunities for pharmacological intervention to manage the function of dopaminergic neurons.

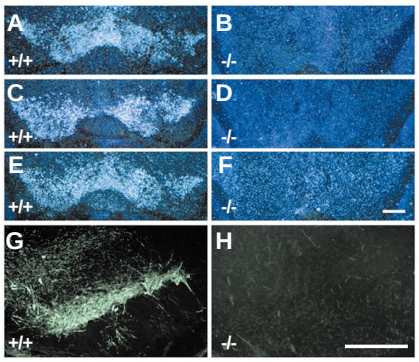

Fig. 3. Markers for DA neurons in the newborn midbrain. In situ hybridization (*22*) (**A** through **F**) and immunohistochemistry (*6*) (**G** and **H**) are shown for coronal sections of wild-type (A, C, E, and G) and Nurr1$^{-/-}$ (B, D, F, and H) mice. Nurr1 was expressed in wild-type (A) (*5*) but not in Nurr1$^{-/-}$ ventral midbrain (B). TH mRNA was detected in wild-type (C) but not in Nurr1$^{-/-}$ (D) ventral-midbrain. c-Ret mRNA was detected in wild-type DA neurons (E) but was virtually absent in Nurr1$^{-/-}$ midbrain (F). Immunohistochemistry of DA neuron marker ADH2 showed labeling in wild-type SNC (G) but not in Nurr1$^{-/-}$ midbrain (H). Scale bars, 300 μm.

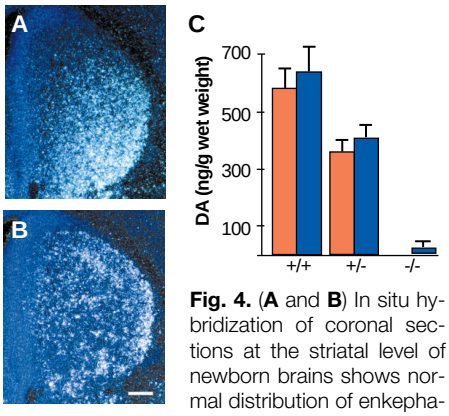

Fig. 4. (**A** and **B**) In situ hybridization of coronal sections at the striatal level of newborn brains shows normal distribution of enkephalin (A) and substance P (B) mRNA in Nurr1$^{-/-}$ mice. Scale bar, 300 μm. (**C**) DA was absent in Nurr1$^{-/-}$ mice and reduced in Nurr1$^{+/-}$ mice. Shown is HPLC analysis (*18*) of dissected striata (orange bars) and ventral midbrains (blue bars) of wild-type (+/+), heterozygote (+/−), and Nurr1 (−/−) mice. The decreases of DA are statistically significant ($P = 0.01$). (DA detected in nigra areas in two of five Nurr1$^{-/-}$ brains in all probability corresponds to contamination from adjacent non-affected DA neurons.)

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6. Embryos and newborn mice were fixed by immersion (4% formaldehyde and 0.4% picric acid), frozen, sectioned, and stained with cresyl violet or prepared for indirect immunohistochemistry [T. Hökfelt, K. Fuxe, M. Goldstein, T. H. Joh, *Histochemie* **33**, 231 (1973)] or according to the ABC kit protocol (Vector Laboratories). Two to four animals of both Nurr1$^{+/+}$ and Nurr1$^{-/-}$ for all stages were used. Antisera and dilutions were TH (Pel-Freez) 1:200 and 1:1000; rodent ADH2 (*9*) 1:200; and Calcitonin gene-related peptide (Penninsula Laboratories), neurofilament, laminin (Bethesda Research Laboratories), and protein gene product 9.5 (Biogenesis), each at 1:400.
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18. Pieces from striatum of newborn wild-type (n = 9), heterozygote (n = 14), and Nurr1$^{-/-}$ (n = 6) mice, and substantia nigra of newborn wild-type (n = 6), heterozygote (n = 12) and Nurr1$^{-/-}$ mice (n = 5) were used for HPLC analysis [see H. Andersson, J. Luthman, E. Lindqvist, L. Olson, *Neurotoxicology* **16**, 201 (1995)]. Pieces from striatum of adult (8 weeks) wild-type (n = 8) and heterozygote (n = 8) mice were used in parallel experiments.
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21. Gene targeting experiments were made essentially as described [A. L. Joyner, Ed., *Gene Targeting: A Practical Approach* (Oxford Univ. Press, Oxford, 1993)]. Genomic Nurr1 sequences were cloned from a mouse 129 genomic library, and sequences (Fig. 1A) were inserted into a targeting vector (pMC1NeoPolyA, Stratagene, La Jolla, CA) that contained a neomycin phosphotransferase gene driven by a herpes simplex virus thymidine kinase gene promoter. Plasmid sequences were excised and the targeting construct was transfected into E14 embryonic stem (ES) cells. G418-resistant clones in which Nurr1 had been targeted were identified by Southern (DNA) blotting, using a 1-kb Nurr1 probe located 5′ of Nurr1 sequences in the targeting construct, ensuring that the authentic Nurr1 locus was detected. ES cells from three independent clones were injected into C57Bl6 recipient blastocysts to obtain germline transmission. The reported phenotype was confirmed with two independent ES cell lines. Resulting animals and embryos were genotyped by means of polymerase chain reactions specific for the wild-type (5′-GTCGGTTTCAGAAGTGC-3′ and 5′-GTAAACGACCTCTCCGG-3′) and targeted allele (5′-CCAATGTCGAGCAAACC-3′ and 5′-CGATCCCCT-CAGAAGAA-3′), respectively.
22. Tissues from 3 (E10.5), 5 (E11.5), and 11 (newborn) mice were used for in situ hybridization with ^{35}S-labeled oligonucleotides [Å. Dagerlind, K. Friberg, A. J. Bean, T. Hökfelt, *Histochemistry* **98**, 39 (1992); (5)]. Sequences of oligonucleotides are available on request.
23. We thank B. Vennström for advice and the generous gift of an ES cell line–derived genomic DNA library and R. Lindahl and D. Dahl for generous gifts of ADH2- and neurofilament antisera, respectively. For excellent technical assistance, we acknowledge A. Foo, E. Nilsson, E. Lindqvist, K. Lundströmer, and K. Nordström. We thank U. Lendahl for advice and R. Pettersson for valuable comments on the manuscript. Supported by the Swedish Medical Research Council and the U.S. Public Health Service.

12 December 1996; accepted 20 February 1997

Regulation of Lipid A Modifications by *Salmonella typhimurium* Virulence Genes *phoP-phoQ*

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Bacterial pathogenesis requires proteins that sense host microenvironments and respond by regulating virulence gene transcription. For Salmonellae, one such regulatory system is PhoP-PhoQ, which regulates genes required for intracellular survival and resistance to cationic peptides. Analysis by mass spectrometry revealed that *Salmonella typhimurium* PhoP-PhoQ regulated structural modifications of lipid A, the host signaling portion of lipopolysaccharide (LPS), by the addition of aminoarabinose and 2-hydroxymyristate. Structurally modified lipid A altered LPS-mediated expression of the adhesion molecule E-selectin by endothelial cells and tumor necrosis factor–α expression by adherent monocytes. Thus, altered responses to environmentally induced lipid A structural modifications may represent a mechanism for bacteria to gain advantage within host tissues.

Pathogenic bacteria coordinately express virulence genes in response to eukaryotic microenvironments (*1*). For many pathogens, this requires sensing and transcriptional activation involving two proteins that form a phosphorelay mechanism. In Salmonellae, one such system comprises a sensor kinase, PhoQ, and a transcriptional activator, PhoP (*2*, *3*). This system can simultaneously activate and repress more than 40 different genes, termed PhoP-activated (*pag*) and PhoP-repressed (*prg*) genes. The *pho-24* allele, as a result of the replacement of amino acid 48 of PhoQ with isoleucine, locks *S. typhimurium* in a state of *pag* activation and *prg* repression termed the PhoP-constitutive phenotype (PhoP^c) (*4*, *5*). Deletion of *phoP* or *phoQ* results in a PhoP null phenotype (PhoP$^-$) (*2*, *3*). Both PhoP^c and PhoP$^-$ bacteria show decreased virulence, which indicates that the ability to sense various mammalian microenvironments and alter gene transcription is essential for pathogenesis (*2*–*5*). PhoP-PhoQ induces transcription of genes essential to virulence in mice, bacterial survival within macrophages, and resistance to cationic antimicrobial peptides (*2*, *3*, *6*, *7*) and represses genes essential for induction of macropinocytosis in macrophages and epithelial cells (*2*, *3*). Genes in the *pag* group are transcriptionally activated within acidified macrophage phagosomes after *S. typhimurium* phagocytosis by cultured macrophages and after infection of mice as measured by in vivo expression technology (IVET) (*8*, *9*). Therefore, PhoP^c bacteria simulate in part the regulation state of bacteria within host tissues and macrophage phagosomes.

LPS is a pathogenic factor of Gram-negative bacteria that consists of three distinct structural regions: O-antigen, core, and lipid A. Both O-antigen and core consist of polysaccharide chains, whereas lipid A is formed primarily of fatty acid and phosphate substituents bonded to a central glucosamine dimer. Lipid A is the major signaling component of LPS that stimulates cytokine release in the host (*10*).

To investigate whether the PhoP-PhoQ system regulated alteration of lipid A structure, we conducted experiments with lipid A and LPS from various *S. typhimurium* strains (*11*). The fatty acid content of LPS and whole bacteria were studied by gas chromatography (GC) and GC–mass spectrometry (MS). Comparison of the molar ratios of C12:0 versus C14:0 fatty acids (Table 1) showed that the wild-type and PhoP$^-$ strains gave a 1:1 ratio, whereas the PhoP^c strain gave a 2:1 ratio. A previously unreported component of *S. typhimurium* LPS, 2-OH C14:0, was observed in the PhoP^c strain in an amount that would make up for the loss of C14:0 (Table 1). Fatty acid profiles from whole bacteria showed that the PhoP^c strain contained 1.6 nmol of 2-OH C14:0 per milligram of cell dry weight, and the molar ratio of 3-OH C14:0 to 2-OH C14:0 for PhoP^c LPS was similar to that of the whole cell. 2-OH C14:0 was not observed in the whole cell of the wild-type and PhoP$^-$ strains, which indicated that the presence of 2-OH C14:0 in LPS from the PhoP^c strain was not an artifact of LPS isolation. In addition, the total quantity of LPS fatty acid (per milligram of dry weight) indicated that the LPS composition differed among wild-type, PhoP^c, and PhoP$^-$ strains (Table 1), which implied that the LPS in the PhoP^c strain contained less O-antigen polysaccharide relative to the

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Science **276** (5310), 248-250.
DOI: 10.1126/science.276.5310.248