

Dopaminergic neuronal differentiation from rat embryonic neural precursors by Nurr1 overexpression

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Abstract

In vitro expanded CNS precursors could provide a renewable source of dopamine (DA) neurons for cell therapy in Parkinson's disease. Functional DA neurons have been derived previously from early midbrain precursors. Here we demonstrate the ability of Nurr1, a nuclear orphan receptor essential for midbrain DA neuron development *in vivo*, to induce dopaminergic differentiation in naïve CNS precursors *in vitro*. Independent of gestational age or brain region of origin, Nurr1-induced precursors expressed dopaminergic markers and exhibited depolarization-evoked DA release *in vitro*. However, these cells were less mature and secreted lower levels of DA

than those derived from mesencephalic precursors. Transplantation of Nurr1-induced DA neuron precursors resulted in limited survival and *in vivo* differentiation. No behavioral improvement in apomorphine-induced rotation scores was observed. These results demonstrate that Nurr1 induces dopaminergic features in naïve CNS precursors *in vitro*. However, additional factors will be required to achieve *in vivo* function and to unravel the full potential of neural precursors for cell therapy in Parkinson's disease.

Keywords: CNS precursor, differentiation, dopamine neuron, Nurr1, Parkinson's disease, tyrosine hydroxylase expression. *J. Neurochem.* (2003) **85**, 1443–1454.

Neural stem cells are defined as a multipotent, self-renewing cell population able to generate the three major CNS lineages: neurons, astrocytes and oligodendrocytes (for review see McKay 1997). There has been great interest in exploiting the potential of CNS stem and precursor cells for brain repair. *In vitro* generation of dopamine (DA) neurons from CNS precursors is of particular interest given over 10 years of clinical experience with fetal tissue transplantation in Parkinson's disease (Olanow *et al.* 1996; Freeman *et al.* 1999; Piccini *et al.* 1999). DA is one of the major neurotransmitters in the CNS and has been implicated in many aspects of brain function, including motor control (Graybiel *et al.* 1994), mood-disorders and addiction (Berke and Hyman 2000) as well as cognition (Nieoullon 2002). DA neurons are localized in specific CNS regions, including the substantia nigra and ventral tegmental area of the midbrain. The loss of midbrain nigral DA neurons is the hallmark pathology in Parkinson's

disease. Recent studies have uncovered a number of genes involved in midbrain DA neuron development, including the

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Abbreviations used: AA, ascorbic acid; AADC, amino acid decarboxylase; bFGF, basic fibroblast growth factor; BrdU, Bromodeoxyuridine; DA, dopamine; DAT, DA transporter; eGFP, enhanced green fluorescent protein; FGF, fibroblast growth factor; GABA, γ -aminobutyric acid; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; HBSS, Hank's balanced salt solution; LGE, lateral ganglionic eminence; MAP2, microtubule-associated protein 2; N-CAM, neuronal cell adhesion molecule; 6-OHDA, 6-hydroxydopamine; RA, all-trans-retinoic acid; SHH, sonic hedgehog; TH, tyrosine hydroxylase; TuJ1, β -tubulin type III; VMAT, vesicular monoamine transporter.

secreted molecules fibroblast growth factor 8 (FGF8) and sonic hedgehog (SHH, Ye *et al.* 1998), and several transcriptional factors expressed during midbrain DA neurons development such as Ptx3 (Smidt *et al.* 1997), Lmx1b (Smidt *et al.* 2000) and Nurr1 (Zetterström *et al.* 1997).

Nurr1 is a member of the nuclear steroid-thyroid orphan receptor superfamily. DA neurons fail to develop in Nurr1 null mutant mice (Zetterström *et al.* 1997; Castillo *et al.* 1998; Saucedo-Cardenas *et al.* 1998). However, the precise role of Nurr1 during DA neuron development remains elusive.

The present study demonstrates that Nurr1 is sufficient for inducing dopaminergic differentiation in CNS precursors from various developmental stages and regions of origin. These findings are in contrast to previous results that suggested the requirement of a yet undefined factor that acts in addition to Nurr1 (Wagner *et al.* 1999) or proposed that Nurr1 solely enhances the expression of tyrosine hydroxylase (TH) without accompanying neural differentiation (Sakurada *et al.* 1999). However, this apparent discrepancy could solely reflect differences in the developmental stage or endogenous growth factor compositions among the various *in vitro* culture systems. In the present study, the yield of Nurr1-derived DA neurons was markedly increased by exposure to ascorbic acid (AA) and B27 supplement. Nurr1-derived DA neurons exhibited spontaneous and potassium-evoked DA release *in vitro* but did not lead to restoration of Parkinsonian symptoms *in vivo*. These data demonstrate the role of Nurr1 in converting naive CNS precursor cells into functional DA neurons *in vitro* as a first step towards the development of CNS precursor based therapies for Parkinson's disease.

Materials and methods

Primary CNS precursor cultures

All animal experiments were carried out according to NIH guidelines. Time-pregnant Sprague–Dawley rats were purchased from Dae Han Biolink (Seoul, Korea). Embryonic CNS tissues were dissected from embryonic day 12 (day of plug = day 0), 14, 16 rat cortex, ventral mesencephalon and lateral ganglionic eminence (LGE; striatal anlage), and mechanically dissociated in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Hank's balanced salt solution (CMF-HBSS; Invitrogen, Carlsbad, CA, USA). Cells were plated in 6-cm tissue culture dishes (Corning, NY, USA) pre-coated with polyornithine/fibronectin [poly-L-ornithine (15 $\mu\text{g}/\text{mL}$, Sigma, St Louis, MO, USA) at 37°C incubator overnight followed by fibronectin (1 $\mu\text{g}/\text{mL}$, Sigma or Invitrogen) for at least 2 h]. Cells were allowed to proliferate in the presence of 20 ng/mL basic fibroblast growth factor (bFGF; R & D Systems, Minneapolis, MN, USA) in serum-free medium (N2, Johe *et al.* 1996). The bFGF-expanded precursor cells were passaged by trypsinization and re-plated onto polyornithine/fibronectin pre-coated coverslips (Carolina Biological Supply Company, Burlington, NC, USA) or 6-cm culture plates for subsequent culture. Cells were kept proliferating in the presence of bFGF for an additional 4–6 days to reach 70–90% confluence before inducing

differentiation by withdrawal of the mitogen. In parallel, E12 ventral midbrain precursors (Studer *et al.* 1998) were proliferated in the presence of bFGF and differentiated directly without prior passaging as a positive control for *in vitro* DA release and *in vivo* function. Except the control E12 cultures, all of the experiments were performed on passaged cultures as described above, unless noted otherwise. Cultures were maintained at 37°C in 5% CO_2 incubator, media changes were carried out every other day, bFGF was supplemented daily. In some studies the following factors were added to the medium: 2% B27 supplement (Invitrogen Corporation, Grand Island, NY, USA), 200 μM ascorbic acid (AA, Sigma), 500 nM all *trans*-retinoid acid (RA, Sigma), 20 ng/mL brain-derived neurotrophic factor, 20 ng/mL glial-derived neurotrophic factor, 20 ng/mL neurotrophin-3 (all from R & D systems).

Primary neuronal culture

Cells were mechanically dissociated from E16 rat cortex and plated at a density of 30 000 cells/ cm^2 on coverslips pre-coated with 25 $\mu\text{g}/\text{mL}$ poly-L-lysine and 10 $\mu\text{g}/\text{mL}$ laminin (Invitrogen). Cells were cultured in Neurobasal medium (Invitrogen) containing 10% fetal bovine serum for 24 h followed by a medium change to serum-free conditions (Neurobasal medium with B27 supplement, 0.5 mM L-glutamine, and 100 U/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin). Cultures were maintained at 37°C in 5% CO_2 incubator for 9 days, medium changes were carried out every 3 days.

Transfection of Nurr1

An expression vector under the control of the EF1 α promoter (pcDNAEF1 α) was constructed by substituting the human cytomegalovirus immediate early promoter of the pcDNA3.1 (+) plasmid (Invitrogen) with the 1.2 kb human EF1 α promoter (Gene bank accession number J04617). pEF1 α Nurr1, a vector expressing Nurr1 under the control of the EF1 α promoter, was constructed by engineering Nurr1 cDNA (kindly provided by Drs James Pickel and Vera Nikodem at NIH, Bethesda, MD, USA) into a multicloning site of the pcDNAEF1 α plasmid. The same procedure was applied to generate a reporter vector pEF1 α eGFP expressing eGFP (enhanced green fluorescent protein; Clontech, Palo Alto, CA, USA). Neural precursors expanded *in vitro* with bFGF for 4 days (60–80% confluence) were cotransfected with 3 molar pEF1 α Nurr1 mixed with 1 molar eGFP vector introduced into the cells using Superfect^R transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Transfection controls were carried out with the same amount of pcDNAEF1 α instead of pEF1 α Nurr1 mixed with the eGFP vector. The cells were differentiated by bFGF withdrawal 1 day after transfection and differentiated for an additional 3 days *in vitro*. The same transfection experiments were also performed in the cortical precursor cultures 4 days after bFGF withdrawal or cultures for post-mitotic neurons (see 'Primary Neuronal Culture'), and transfected cells were analyzed 3 days after transfection. The transfected cells were subsequently visualized by direct microscopic examination using a green fluorescent protein (GFP) filter (Nikon, Tokyo, Japan) or by immunohistochemical detection of the GFP protein.

Retroviral construction and transduction

The full-length Nurr1 cDNA was amplified with primers 5' XbaI (5'-AAAATCTAGACCGCCACCATGGATATGCACTGCAAAGC-

AGAC-3') and 3' BglII (5'-AAAAGATCTTTAGAAAGGTAAGG-TGTCCAGGAAAAGTTTG-3') using high fidelity *Taq* polymerase (Biotools, Madrid, Spain). The PCR products were digested with *Xba*I and *Bgl*II restriction enzymes and cloned into corresponding sites in MFG retroviral vector by replacing the thymidine kinase (TK) sequence of MFG.TK.IRES.GFP (Oh *et al.* 2001). The retroviral plasmids were introduced into 293gpg retrovirus packaging cell line (Ory *et al.* 1996) by transient transfection with Lipofectamine (Invitrogen). After 72 h, the supernatants were harvested and used for retroviral infection. In parallel, retroviruses expressing GFP using MFG.GFP sequence were produced as a control (mock transduction).

Neural precursors expanded with bFGF *in vitro* for 4 days were incubated with the viral supernatant containing polybrene (4 µg/mL) for 4 h, followed by a medium change. The following day, precursors were differentiated by bFGF withdrawal and maintained under differentiation conditions for an additional 2–5 days. For a clonal analysis experiment, bFGF-expanded precursors (unpassaged) were incubated with the viral supernatant for 2 h as described above, then the cells were dissociated, harvested and incubated again with the viral supernatant in a suspension for 2 h, and then followed by the procedure for clonal analysis.

Clonal analysis

The cells, transduced twice as described above, were plated at 2000–5000 cells per 10-cm dish and allowed to settle for 2 h. Isolated single cells were marked on the bottom of the plates with a 3-mm circle (Nikon) and cells that grew within the marked circles were referred to as clones. Cells were expanded to form clones for 4 days with bFGF and then induced to differentiate. Nurr1-transduced clones were marked again by estimating GFP expression with an observation on a GFP-filtered microscope 5 days after differentiation (the Nurr1 retroviral vector is designed for GFP to be expressed using the IRES-GFP sequence in the infected cells; see 'Retroviral construction and transduction'). At this stage, 30–70% of the clones expressed GFP. The Nurr1-transduced clones were cultured two more days and analyzed. The identical experiment was, in parallel, performed using the cells infected with GFP-expressing retrovirus as a control.

Immunocytochemistry

Cultured cells were fixed in 4% paraformaldehyde/0.15% picric acid in phosphate-buffered saline [for γ -aminobutyric acid (GABA) immunostaining, 0.2% glutaraldehyde (Sigma) was included in the fixative] and were incubated with primary antibodies overnight at 4°C. The following primary antibodies were used at the concentrations given: Nurr1 polyclonal 1 : 200 (Chemicon, Temecula, CA, USA), nestin polyclonal #130 1 : 50 (Martha Marvin and Ron McKay, National Institute of Health, Bethesda, MD, USA), TH polyclonal 1 : 250 (Pel-Freez, Rogers, AR, USA) or monoclonal 1 : 1000 (Sigma), DBH monoclonal 1 : 100 (Pharmingen, San Diego, CA, USA), GABA polyclonal 1 : 700 (Sigma), β -tubulin type III (TuJ1) monoclonal 1 : 500 or polyclonal 1 : 2000 (both Babco, Richmond, CA, USA), microtubule-associated protein 2 (MAP2) monoclonal 1 : 200 (Sigma), neuronal cell adhesion molecule (N-CAM) polyclonal 1 : 1000 (Chemicon), Glial fibrillary acidic protein (GFAP) monoclonal 1 : 400 (DAKO, Glostrup, Denmark), Bromodeoxyuridine (BrdU)

polyclonal 1 : 100 (Accurate Chemical & Scientific corporation, Westbury, NY, USA) and GFP monoclonal 1 : 400 (Roche Molecular Biochemicals, Basel, Switzerland). For detection of primary antibodies, fluorescence labeled (FITC or Cy3) secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA, USA) were used according to the specifications of the manufacturer. Cells and tissue sections were mounted in VECTA-SHIELD^R with DAPI (Vector Laboratories, Burlingame, CA, USA) mounting medium for fluorescence and photographed using a fluorescent microscope (Nikon).

BrdU incorporation

One day after retroviral infection, BrdU (10 µM) was added to cultures for 1 h just prior to fixation. Anti-BrdU staining was performed as described above.

RNA extraction and reverse transcription-PCR (RT-PCR) analysis

Total cellular RNA was prepared using TRI REAGENT (Molecular Research Center, Inc. Cincinnati, OH, USA) according to the recommendations of the manufacturer. Superscript kit (Invitrogen) was used for the cDNA synthesis. The PCR reactions were carried out according to standard protocols. Optimal MgCl₂ concentrations and cycle numbers for linear amplification range were determined. Primer sequences (forward and backward) and product sizes (base pairs) were as follows: GAPDH (5'-GGCATTGCTCTCAAT GAC-AA-3', 5'-AGGGCCTCTCTTGTCTC-3', 165); TH (5'-TGTC-AGAGGAGCCCGAGGTC-3', 5'-CCAAGAGCAGCCCATCAA-AG-3', 300); DAT (5'-GGACCAATGTTCTTCAGTGGTGGC-3', 5'-GGATCCATGGGAGGTCCATGG-3', 511); VMAT (5'-ATCCA-GACCACCAGACCAGAG-3', 5'-CCCCATCCAAGAGCACCAA GG-3', 616); AADC (5'-CCTACTGGCTGCTCGGACTAA-3', 5'-GCGTACCAGTGACTCAAAC-3', 715); Ptx3 (5'-CGTGCG-TGGTTGGTTCAAGAAC-3', 5'-GCGGTGAGAATACAGGTTG-TGAAG-3', 257); c-ret (5'-GCTGATGCAATGGGCGGCTTGT-GC-3', 5'-GCGCCCCGAGTGTGAGGAATGTGG-3', 541); En1 (5'-TCAAGACTGACTACAGCAACCC-3', 5'-CTTTGTCTCGA-ACCGTGGGTGGTAG-3', 381); Pax5 (5'-CAGATGATAGTCCGC-CAAAGGATAG-3', 5'-ATGCCACTGATGGAGTATGAGGAG-CC-3', 451); Pax2 (5'-CCAAAGTGGTGGACAAGATTGCC-3', 5'-GGGATAGGAAGGACGCTCAAAGAC-3', 545); SHH (5'-GGAAGATCACAAGAACTCCGAC-3', 5'-GGATGCGAGC-TTTGGATTCATAG-3', 354); Smo (5'-TGCTGTGTGCTGTCTAC-ATGCC-3', 5'-TCTTGGGGTTGTCTGTCTCTAC-3', 240); FGF8 (5'-CATGTGAGGGACCAGAGCC-3', 5'-GTAGTTGTTCTCCA-GCAGGATC-3', 312); FGFR3 (5'-ATCCTCGGGAGATGACGAA-GAC-3', 5'-GGATGCTGCCAACTTGTCTC-3', 326).

RT-PCR products were analyzed in an agarose gel containing ethidium bromide.

Measurement of neurite outgrowth

The basic pattern of neurite outgrowth was analyzed as previously described (Oh *et al.* 1996). Briefly, TH⁺ cells from randomly selected areas of at least five cultures from three independent experiments were photographed. Morphological characteristics were quantitated using an Axiovert Phase-Contrast Microscope equipped with an AxioCam digital camera system and Axiovision image analyzer (Carl Zeiss, Jena, Germany). Clusters of cells were

excluded from the morphometric analysis. The length of primary neurite was defined as the distance from the soma to the tip of the longest branch. The total extent of neurite was defined as the combined lengths of all neurites per cell. The number of neurites per cell was identified as all processes longer than two cell diameters in length.

DA determination by reverse-phase HPLC

DA releases were determined in the cultures for precursors after 5 days of differentiation in conditioned medium (48 h after last medium change), in HBSS (15 min, basal release), and in HBSS plus 56 mM KCl (15 min, evoked release). DA in the medium was immediately stabilized with orthophosphoric acid (7.5%)/metabisulfate (0.22 mg/mL) and stored at -80°C until analysis (Studer *et al.* 1996). After centrifugation at 1500 *g* through centrifugal filter devices (Microcon YM-10, Millipore Co., MA, USA) to eliminate proteins and other macromolecular constituents from the sample, DA was extracted by aluminum adsorption and eluted in 0.1 M perchloric acid. The DA was separated by reverse phase μ -Bondapak C18 column (300 \times 3.9 mm, Waters, Cotland, NY, USA) that was maintained at 32°C with a column heater (Waters). The mobile phase consisted of 0.05 M citric acid, 0.05 M disodium phosphate (pH 3.1), 3.2 mM 1-octanesulfonic acid (sodium salt), 0.3 mM EDTA and 12% methanol, and was pumped at a flow rate of 0.5 mL/min using a solvent delivery system (Waters). Samples (20 μL) were injected with a Rheodyne injector and the compounds were analyzed using a coulometric detector (ESA, Bedford, MA, USA) with an analytical cell (Model 5014B, ESA). The potential for the first and second cell was set at +10 mV and +320 mV, respectively. A guard cell (ESA, potential +450 mV) was placed before the injector. The concentrations of DA detected in the supernatants were determined by comparison with a standard solution of DA injected into the column immediately before and after each experiment.

In vivo studies

Male Sprague–Dawley rats, weighing 220–250 g at the start of the experiment, were used for all the *in vivo* study. Under pentobarbital anesthesia, 4 μL of 6-hydroxydopamine (6-OHDA, 3 $\mu\text{g}/\mu\text{L}$ in normal saline containing 0.2 mg/mL AA) was injected stereotactically into the substantia nigra and the median forebrain bundle (4.8 and 1.8 mm posterior to bregma; 1.5 and 1.8 mm lateral to midline; 8.2 and 8.0 mm ventral to the dura). The incisor bar was set at 3.5 mm below zero (interaural line).

Three weeks after the 6-OHDA lesioning animals were tested for apomorphine-induced turning behavior (apomorphine at 0.2 mg/kg *i.p.* in saline containing 2 mg/mL AA). The rats were tested over a 30 min interval and animals with net rotational asymmetry of at least 10 full turns per minute away from the lesioned side were selected for transplantation surgery. The apomorphine-induced rotation was evaluated at 1, 2, 3, 4, 5 and 6 weeks after transplantation.

At 2 days after differentiation, the cells were dissociated by trypsinization and suspended in normal saline. Animals were anesthetized with pentobarbital and placed in a stereotaxic frame. Using an 18-gauge needle, 3 μL of the cell suspensions (1×10^5 cells/ μL) were injected over a 5 min period in the lesioned striatum at each of the three sites (0.2, 0.2, 1.2 mm anterior to

bregma; 3.0, 3.0, 2.5 mm lateral to midline; 5.5, 4.0, 4.7 mm ventral to the dura, setting the incisor bar at 3.5 mm below interaural zero). The needle was left in place for 3 min following the completion of each injection. The rats received daily injections of cyclosporine A (10 mg/kg, *i.p.*) starting 1 day before grafting and continuing for 20 days.

Six weeks after transplantation, animals were anesthetized (50 mg/kg phenobarbital) and intracardially perfused with 4% paraformaldehyde in phosphate-buffered saline. Brains were removed and immersed in 30% sucrose in phosphate-buffered saline overnight and sliced on a freezing microtome (CM 1850, Leica, Wetzlar, Germany). Free-floating brain sections (40 μm thick) were subjected to TH-immunohistochemistry as described above and image was captured using a confocal microscope (LSM510, Zeiss, Feldbach, Switzerland).

Cell counting and statistical analysis

Immunoreactive or DAPI-stained cells were counted in 5–10 uniform randomly chosen areas of each well using an eye piece grid at a final magnification of 200 or 400. Three to six culture wells were analyzed in each experiment. Data are expressed as mean \pm SEM. Statistical comparisons were made by ANOVA with Tukey *post hoc* analysis (SPSS 11.0; SPSS Inc., Chicago, IL, USA) when more than two groups were involved.

Results

Nurr1 transfection induced TH expression in cortical CNS precursors

Neural precursors were expanded and passaged in serum-free medium in the presence of bFGF (see 'Material and methods'). After bFGF-proliferation for additional 4–6 days, over 95% of cells was immunoreactive for the intermediate filament nestin, a marker of neural precursor cells. Less than 3% of the cells expressed neuronal (TuJ1) or astroglial (GFAP) markers. None of the nestin + cells was positive for TuJ1 and less than 0.5% coexpressed GFAP. The similar proportions of these immunoreactive cells were observed in the passaged cultures for neural precursors regardless of embryonic ages (E12–E16) and regions of origin [cortex, midbrain, LGE] from which neural precursors were isolated. These findings demonstrate the selectiveness of the culture conditions for neural precursors.

To examine the role of Nurr1 in DA differentiation, bFGF expanded and passaged E14 cortical precursors were transfected with constitutively expressed Nurr1 (pEF1 α -Nurr1). Co-transfection with an eGFP-expressing vector was used to identify Nurr1-overexpressing cells. One day after transfection, precursors were differentiated by bFGF withdrawal and differentiation for another 3 days. Very few (< 0.01%) TH+ cells were detected in control precursor cultures transfected with eGFP only. Nurr1 transfection caused a dramatic increase in the number of TH+ cells (Fig. 1). Almost all TH immunoreactivity was detected exclusively in the trans-

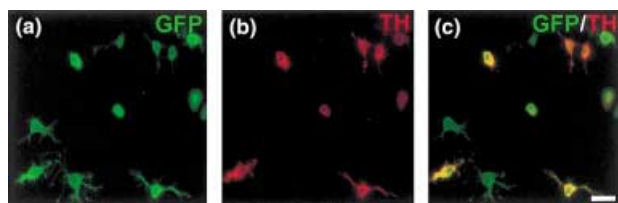


Fig. 1 Nurr1-induced TH expression in the cultures for E14 cortical precursors. Cortical precursors were cotransfected with a Nurr1-(pEF1 α Nurr1) and a GFP-expressing vector (pEF1 α GFP) and the transfected cells were visualized by immunostaining against GFP protein. The differentiation of the transfected precursors was induced by withdrawal of bFGF at the following day of transfection. Three days after differentiation, double immunocytochemical analyses for TH (b) and GFP (a) were performed in the transfected cultures. TH+/GFP+ cells are yellow in the merged image of TH and GFP immunostaining (c). TH immunoreactivities (TH+/GFP+, yellow in c) were detected in the Nurr1 transfected cells, whereas no TH cell was shown in the cultures transfected with the control vector (pcDNAEF1 α , data not shown). Scale bar, 20 μ m.

fected (GFP+) cells and no TH immunoreactivity was observed in untransfected cells. This suggests that cell autonomous expression of Nurr1 is required for TH induction in these precursors. The percentage of GFP+ cells expressing TH was $15 \pm 2.5\%$ in Nurr1 transfected cultures vs. 0% in control cultures ($n = 10$). TH+ cells did not coexpress dopamine β -hydroxylase, a marker of noradrenergic or adrenergic neurons (data not shown), confirming their dopaminergic identity. Four days after bFGF withdrawal, over 70% of cells were positive to TuJ1 or GFAP. Nurr1 transfection into the cell differentiated for 4 days did not induce TH expression. Similarly, Nurr1 was unable to induce TH+ cell in primary neuronal cultures containing >90% post-mitotic neurons. These findings indicate that the DA-inducing effect of Nurr1 specifically occurs during precursor proliferation but not in differentiated neurons or astrocytes.

Efficient generation of TH+ cells by retroviral Nurr1 transduction in combination with AA and B27 treatment

Due to the low transfection efficiency using liposome-mediated gene transfer (< 0.5% of total cells), a Nurr1 retroviral expression vector was generated to efficiently transduce proliferating E14 cortical precursor cells. Upon 2 days of retroviral infection, $44.9 \pm 1.6\%$ of total cells ($n = 30$) were immunoreactive for Nurr1 in transduced cultures, whereas no Nurr1+ cells were detected in untransduced control cultures. Retroviral gene transfer led to the efficient generation of TH+ cells (Fig. 2a). One day after infection, 1.5% of the cells were TH+ (the day immediately before bFGF-withdrawal). The number of TH+ cells gradually increased up to 5 days after bFGF-withdrawal (8.1% , $n = 30$) and was maintained for at least up to 10 days of differentiation. Whereas the effect of AA on dopaminergic

differentiation has been demonstrated previously (Yan *et al.* 2001; Lee *et al.* 2003), exposure to B27 supplement also increased DA neuron differentiation in midbrain precursors (data not shown). Here we tested whether AA and B27 might further enhance DA neuron differentiation in Nurr1-induced cortical precursors. Supplementation of the medium with either AA or B27 led to a dramatic increase in TH+ cells and the addition of both factors showed an additive effect (Fig. 2a). Exposure to AA and B27 during culture period after Nurr1 transduction increased the number of TH+ cells derived from Nurr1-transduced cortical precursors from 8.1% (see above) to 23.3 ± 1.0 and $31.5 \pm 1.7\%$ TH+ cells, respectively, 5 days after differentiation (6 days after infection). Combined treatment with AA and B27 resulted in $37.5 \pm 1.1\%$ TH+ cells out of the total cells population ($n = 40$ for all values, $p < 0.01$ for combined treatment vs. AA and $p < 0.05$ for combined treatment vs. B27). On the other hand, either AA or B27 by itself did not elicit any effect of increasing TH+ cells in untransduced cultures (Fig. 2a), although both AA and B27, as described above, have the effect in the cultures for midbrain precursors from which a certain proportion of TH+ cells are normally generated (Yan *et al.* 2001). These data suggest that AA and B27 act in an additive fashion on the Nurr1-induced generation of TH+ cells. Conversely, differentiation into GABAergic neurons was significantly decreased in Nurr1-transduced cultures ($9.0 \pm 2.9\%$ in Nurr1 transduced vs. $31.7 \pm 3.9\%$ in control cultures (both supplemented with AA and B27; $n = 40$, $p < 0.01$) (Fig. 2b).

The yield of Nurr1-induced TH+ cells was not increased by other known neurotrophic factors such as brain-derived neurotrophic factor, glial-derived neurotrophic factor, neurotrophin-3, or exposure to RA.

The Nurr1-mediated induction of TH+ cells were not limited to the precursors derived from the E14 cortex. Neural precursors isolated from E12–E16 as well as precursors from other regions such as the midbrain or LGE displayed similar responses (Table 1).

Nurr1-induced TH+ cells adopt DA neuronal fate upon differentiation

Similar to the transfection results, all TH+ cells colocalized Nurr1 (Fig. 3e), and $79.1 \pm 3.8\%$ of all Nurr1+ cells were TH+ 5 days after differentiation ($n = 25$). Nurr1-induced TH+ cells exhibited two distinct morphologies: (i) TH+ cells typically arranged in clusters displaying immature precursor morphologies (Fig. 3b, and arrow in Fig. 3a) and coexpressing nestin (Fig. 3f) and (ii) TH+ cells exhibiting typical neuronal morphologies (Figs 3c and d and arrowhead in Fig. 3a) and expressing TuJ1 ($23.2 \pm 1.1\%$ of TH+ cells, $n = 20$; arrow in Fig. 3g), MAP2 ($11.8 \pm 1.3\%$ of TH+ cells, $n = 20$; arrow in Fig. 3h), and N-CAM (> 95% of TH+ cells; Fig. 3i). TH+ cells without TuJ1 or MAP2 expression (arrowhead in Figs 3g and h), presented with

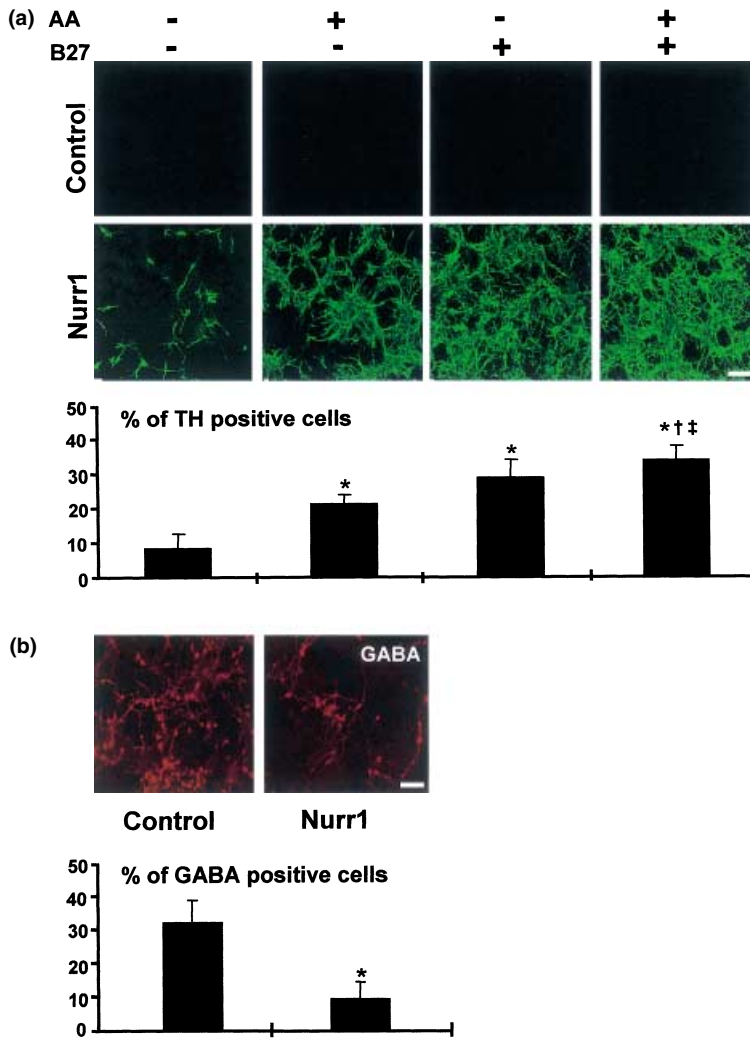


Fig. 2 Efficient generation of TH-positive cells from cortical precursors by Nurr1 overexpression using retroviral infection. Precursors from rat E14 cortices were expanded for 4 days in the presence of bFGF and then subjected to infection with Nurr1-recombined retroviruses. The differentiation of the Nurr1-infected precursors was induced by withdrawal of bFGF at the following day of infection, and further cultured for 5 days in the absence or presence of AA and B27 supplement. (a) Immunocytochemical analyses for TH and yields of TH+ cells in each treatment. Nurr1 transduction efficiently generates TH+ cells, and the yield was markedly improved by AA and B27 treatment. Significantly different from untreated control (*) at $p < 0.01$, AA-treated (†) at $p < 0.01$, and B27-treated (§) at $p < 0.05$. On the contrary, the number of GABA+ cells were significantly reduced by the Nurr1 in the cultures supplemented with AA and B27 (b). Significantly different from untreated control (*) at $p < 0.01$. Scale bar, 20 μ m.

Table 1 The yields of TH+ cells in Nurr1-transduced cultures for CNS precursors isolated from a variety of embryonic ages or brain regions

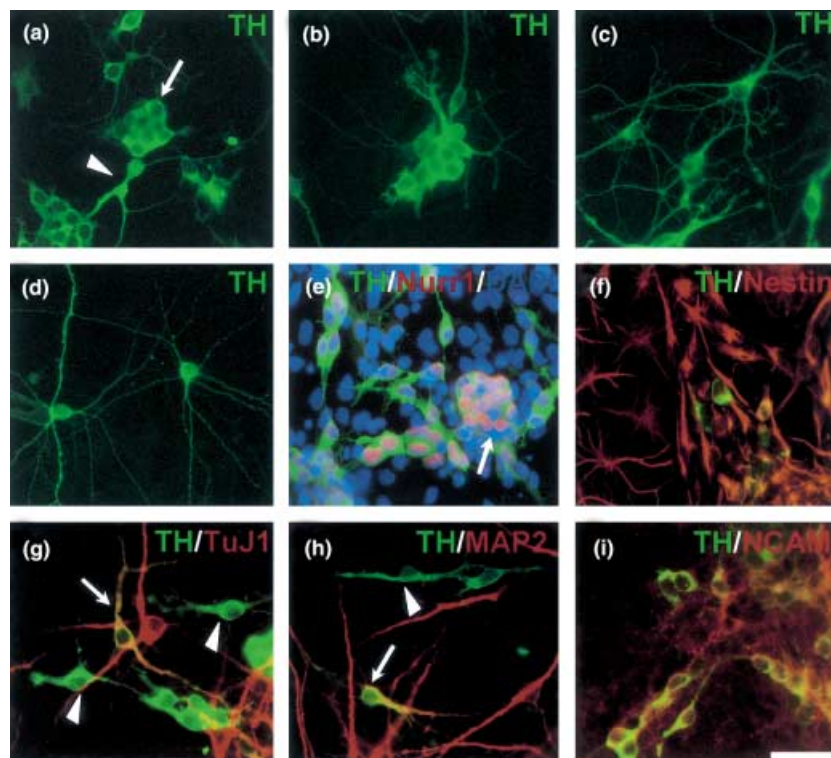
Nurr1-transduced culture (%)									Untransduced culture (%)							
Cortex			Midbrain			LGE			Cortex			Midbrain			LGE	
E12	E14	E16	E12	E14	E16	E14	E16		E12	E14	E16	*E12	E14	E16	E14	E16
20.1 \pm 1.2	28.8 \pm 3.5	33.2 \pm 5.1	27.0 \pm 3.8	14.4 \pm 1.0	17.7 \pm 1.1	14.2 \pm 4.3	17.0 \pm 3.1	< 0.01	ND	ND	ND	0.7 \pm 0.2	ND	ND	ND	ND

*Although bFGF-expanded, but unpassaged E12 mesencephalic precursors efficiently differentiate into TH+ cells (8–18% of the total cells), the yield of TH+ cells steeply decreases if the precursors were subcultured (Yan *et al.* 2001). All of the data presented were obtained from the passaged cultures (see 'Experimental methods') supplemented with AA. Data represent the mean \pm SEM from two independent experiments ($n = 35$ for all values). ND, not detected.

the levels of TH immunoreactivity. Among the TH+ cells with precursor morphologies, there was a subgroup that was negative for nestin and whose identity remains undetermined. These results indicate that not all Nurr1-induced TH+ cells acquire neuronal characteristics. Longer

in vitro differentiation or treatment with RA or wide range of neurotrophic factors did not affect the number of TH+ cells expressing neuronal markers. No colocalization of TH and GFAP was detected in any of the experiments. This suggests that Nurr1 induces TH expressing in neural

Fig. 3 Morphological and immunological properties of Nurr1-induced TH+ cells. (a) Nurr1-induced TH+ cells have two kinds of shapes. TH+ cells detected in the clusters were rounded immature precursor-shaped (b and arrow in a), and the others have a typical neuronal morphology (c, d and arrowhead in a) after 3 days of differentiation. Among these cells, a portion of TH+ cells shows highly mature neuronal morphologies (c and d). (e) Nurr1 was colocalized in the TH+ cells generated by exogenous Nurr1, especially in those of clusters (arrow in e). (f) Nestin, an intermediate filament specific for neural precursors, was colocalized in the TH+ cells with immature morphology. (g, h and i) Colocalization of neuronal markers in the TH+ cells. TuJ1 (arrow in g), MAP2 (arrow in h), and N-CAM (i) were colocalized in the Nurr1-TH cells. A portion of Nurr1-TH+ cells with classic neuronal shapes (arrowhead in g and h), however, did not double label with TuJ1 or MAP2. Scale bar, 20 μ m.



precursors undergoing neuronal but not astrocytic differentiation.

RT-PCR analyses demonstrated Nurr1-induced expressions of genes involved in DA synthesis and transport including TH, L-aromatic amino acid decarboxylase (AADC), DA transporter (DAT) and vesicular monoamine transporter (VMAT), as well as the midbrain DA neuron marker (Ptx-3) without affecting c-ret, a gene reported to be Nurr1-dependent (Wallen *et al.* 2001). No Nurr1-dependent changes were observed in the expression of genes controlling early midbrain development such as SHH, Smoothened (Smo), FGF8, FGF receptor 3 (FGFR-3), Engrailed-1 (En1), Pax2, and Pax5 (Fig. 4b). Expression pattern of Nurr1-transduced E14 midbrain cells was not different from that of cortical cells.

DA release in Nurr1-induced TH+ neurons

Synaptic release of the neurotransmitter DA is a definitive measure for the identification of DA neurons. We evaluated the DA releases from E14 cortical and E14 midbrain precursors transduced with Nurr1. Cells were differentiated for 5 days, and conditioned medium (48 h after last medium change) was collected and tested by HPLC. DA was readily detected in the conditioned medium prepared from Nurr1-transduced cultures (808.9 ± 61.3 , $n = 5$ and 1336.9 ± 82.4 , $n = 6$, pg/ 10^6 TH+ cells of DA in E14 cortical and midbrain cultures, respectively). Basal DA release (15 min HBSS)

yielded 334.9 ± 50.5 , 253.0 ± 17.4 pg/ 10^6 TH+ cells in cortical and midbrain-derived precursors, respectively. KCl-evoked release (15 min 56 mM KCl) was 1709.1 ± 117.9 and 1947.3 ± 136.3 pg/ 10^6 TH+ cells ($p < 0.05$, paired *t*-test, comparing evoked to basal release). No norepinephrine and epinephrine release could be detected. These data provide *in vitro* functional evidence of Nurr1-induced DA neuron identity.

Morphological differentiation of Nurr1-induced TH+ neurons

Nurr1-induced DA neurons exhibit less mature morphologies than DA neurons derived from E12 midbrain precursors (Fig. 5c), though a small subset of Nurr1-DA displays highly elaborated morphologies (Figs 3c and d). Morphometric analyses compared length and number of dendrites 5 days after differentiation. As shown in Table 2, number of neurites, length of the primary neurite, and total neuritic extent were significantly increased in E12-DA neurons compared with those in Nurr1-DA cells. Consistent with these morphological observations, E12 precursor-derived DA neurons secreted 10–20 folds more DA than Nurr1-induced DA neurons (Fig. 5b). DA levels in the E12 precursor-derived TH+ cells were 17.0 ± 2.3 , 5.4 ± 3.4 , and 19.1 ± 5.3 ng/ 10^6 TH+ cells in conditioned medium, basal and evoked release, respectively ($n = 5$ for all values).

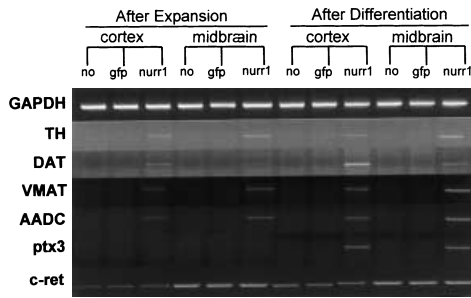
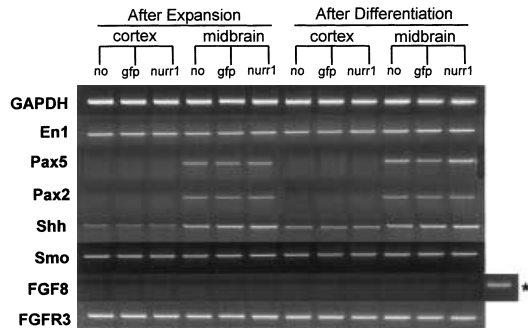
(a) DA phenotype**(b) Midbrain DA development**

Fig. 4 RT-PCR analyses for the genes specific to DA neuronal phenotype (a) and midbrain DA development (b). The expression was assessed after 4 days of expansion and 6 days of differentiation in the cultures for E14 cortical (lanes 1–3 and 7–9) and E14 midbrain (lanes 4–6 and 10–12) precursors. Lanes 1, 4, 7 and 10, untransduced control; lanes 2, 5, 8 and 11, GFP-transduced control; lanes 3, 6, 9 and 12, Nurr1-transduced. Nurr1-dependent gene expressions were observed in the genes specific to general dopaminergic (TH, AADC, DAT and VMAT) and midbrain-specific dopaminergic phenotypes (Ptx-3), whereas the expression of the candidate genes involved in midbrain DA neuronal development was not altered by the Nurr1 overexpression. *Positive control, cDNA isolated from E14.5 midbrain tissue including isthmus.

Effect of Nurr1 on neuronal and astrocytic differentiation of cortical precursors

Nurr1-overexpression did not affect the proliferation rate in neural precursors or the ratio of astrocytic vs. neuronal progeny upon cell differentiation. BrdU incorporation studies during bFGF proliferation (1 h BrdU exposure immediately prior to fixation) yielded $29.2 \pm 1.7\%$ and $29.8 \pm 1.9\%$ in S phase for Nurr1-transduced and control cultures, respectively ($n = 20$ each). The percentages of TuJ1+ cells in cortical precursors 5 days after bFGF withdrawal were $43.9 \pm 2.7\%$ in Nurr1-transduced cultures vs. $45.3 \pm 4.3\%$ in GFP-transduced control cultures. GFAP percentages were 36.6 ± 2.8 and 41.1 ± 2.2 in Nurr1 transduced and control cultures, respectively ($n = 20$ each).

Clonal analysis was performed to study the effect of Nurr1 on single cortical precursors (see 'Experimental methods').

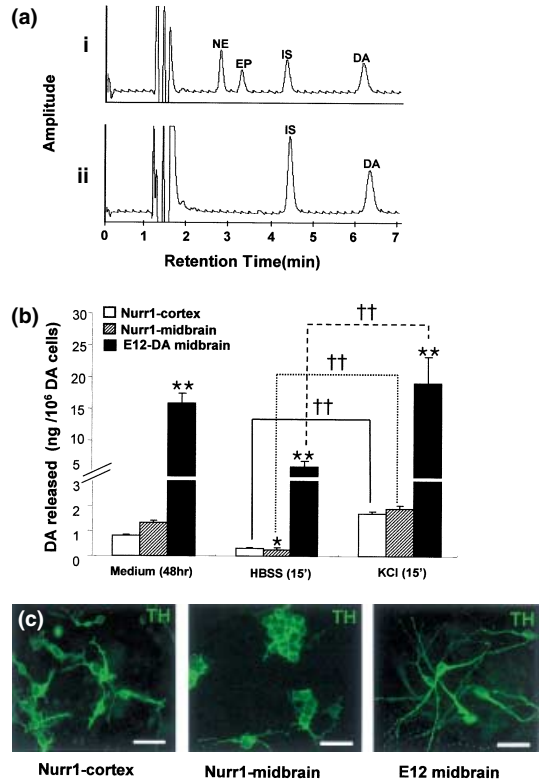


Fig. 5 Comparison of morphology and *in vitro* DA release between Nurr1-induced and normally *in vitro*-differentiated DA neurons. (a) (i) Chromatogram for mixture of standards (NE, norepinephrine; EP, epinephrine; and DA). IS represents the internal standard (3,4-dihydroxybenzylamine) used for quantification of DA concentrations. (ii) Typical chromatogram for DA in the conditioned culture medium. (b) HPLC quantification of DA in conditioned medium (48 h), released in HBSS for 15 min, and released in HBSS with 56 mM KCl for 15 min. After 5 days of differentiation, DA releases were determined in each condition in the cultures for Nurr1-infected E14 cortical, midbrain precursors and for E12 midbrain precursors normally differentiated *in vitro*. In all the cultures, DA releases were evoked by the potassium-induced depolarization stimuli. Consistent with the maturities in the neuronal morphology shown in (c) and Table 2, DA neurons differentiated from E12 midbrain secrete 10–20-fold more DA compared to the Nurr1-induced DA neurons. †Significantly different from Nurr1-cortical DA in ANOVA test. **, Significantly different from basal release (HBSS) in paired *t*-test. * $p < 0.05$; ††, ** $p < 0.01$. Scale bar, 20 μ m.

Single isolated cells were proliferation for 4 days and differentiated progeny of the clones was expanded after 7 days of bFGF withdrawal. At the end of culture, the average clone sizes were 477 ± 51 and 528 ± 87 cells in Nurr1- and control GFP-transduced clones, respectively ($n = 51$, four independent experiments, $p = 0.638$). No difference in the proportion of astrocytes to neurons was observed in Nurr1-transduced vs. control cultures. There was a subgroup of clones that did not yield either TuJ1+ or

Table 2 Comparison of neurite outgrowth between E12-DA and Nurr1-DA neurons

	E14 Nurr1-cortex (<i>n</i> = 50)	E14 Nurr1-midbrain (<i>n</i> = 90)	E12 midbrain (<i>n</i> = 50)
Primary neurite (μm)	15.56 \pm 0.89	10.29 \pm 0.81*	105.30 \pm 7.2*
Total neurite extent (μm)	25.72 \pm 1.54	17.12 \pm 1.33*	337.10 \pm 14.5*
Number of neuritis per cell	2.02 \pm 0.09	2.16 \pm 0.07	4.35 \pm 0.29*

The measurements were performed in randomly selected TH+ cells 5 days after differentiation. Data are presented as mean \pm SEM. *Significantly different from the values of Nurr1-cortical DA neurons at $p < 0.001$.

GFAP+ cells. These clones contained precursors only (undifferentiated clones: $21.5 \pm 6.5\%$ of all clones in Nurr1-transduced vs. $20.4 \pm 6.1\%$ in GFP-transduced group). The proportions of the clones producing neuron and astrocyte (dipotent), neuron-only and astrocyte-only in the Nurr1-transduced cultures were not significantly different from the GFP-transduced control cultures: $35.2 \pm 7.6\%$ vs. $42.8 \pm 6.3\%$ (dipotent clone), $37.2 \pm 6.9\%$ vs. $30.6 \pm 2.8\%$ (neuron-only clone), and $5.8 \pm 1.4\%$ vs. $6.1 \pm 1.1\%$ (astrocyte-only clone) in Nurr1-transduced vs. GFP-transduced control cultures, respectively ($n = 4$ for each value; 61 Nurr1 clones and 59 control clones were analyzed from four independent experiments). Absolute numbers of neurons and astrocytes also did not differ between Nurr1 and control clones (data not shown). These findings, collectively, suggest that Nurr1 overexpression does not interfere with proliferation or neuronal and astrocytic differentiation of neural precursors.

TH expression in differentiated clones confirmed our results with non-clonal cultures. TH+ cells were detected in $72.9 \pm 3.6\%$ ($n = 3$) of the Nurr1-clones and $37.3 \pm 10.6\%$ ($n = 22$ from three independent experiments) of the cells in the TH-producing clones were positive to TH. This result demonstrates that single cortical precursor cells can give rise to TH+ cells after Nurr1 transduction without the need for any extrinsic midbrain derived cofactors as proposed previously (Wagner *et al.* 1999).

Apomorphine-induced rotational behavior

Cells derived from Nurr1-transduced E14 cortical and E14 midbrain precursors were grafted into the ipsilateral striatum of 6-OHDA-lesioned rats. A previous study has shown the effective reduction of amphetamine-induced rotation scores in the animals grafted with precursors isolated from E12 rat midbrain (Studer *et al.* 1998). In accordance with this previous work, transplantation of unpassaged E12 midbrain precursors (2 days after differentiation) led to reduced rotation scores 6 weeks after transplantation [out of seven animals grafted, three showed a marked ($> 80\%$) and two animals a slight ($> 30\%$) reduction]. The average percentage decrease in rotation scores as compared to pre-transplantation values were 7.0 ± 8.4 , 17.2 ± 10.7 , 25.0 ± 10.2 , 27.8 ± 14.0 , 34.3 ± 13.7 and $47.4 \pm 13.8\%$ after 1, 2, 3, 4, 5 and 6 weeks

of transplantation ($n = 7$). However, no reduction of apomorphine-induced rotation scores was observed in animals grafted with Nurr1-transduced cortical or midbrain precursors ($n = 8$ for the cortical and $n = 7$ for midbrain precursors) (Fig. 6a). Consistent with poor behavioral results, histology 6 weeks after transplantation revealed fewer surviving TH+ cells in the Nurr1-transduced group, compared with E12 precursor-grafted group: 2422.5 ± 212.7 (Nurr1-transduced E14 cortical precursors) and 3120.0 ± 196.9 (Nurr1-transduced E14 midbrain precursors) vs. 8895.0 ± 365.1 in the E12-precursor group, $n = 3$ for all values, corresponding to about 4, 5 and 18% of TH+ cell survival rates of total TH+ cells grafted, respectively. Furthermore, TH+ cells in the grafts with E12 midbrain precursors appeared much more differentiated with numerous long processes extending into the host striatum (iii and vi in Fig. 6b), whereas TH cells in the Nurr1-transduced groups exhibited immature neuronal morphologies (i, iv and ii, v in Fig. 6b).

Discussion

The efficient *in vitro* generation of DA neurons from CNS precursors is of great interest due to the great clinical need for a renewable cell source in experimental transplantation studies in Parkinson's disease. Several studies have attempted DA neuron conversion of neural stem and precursor cells via overexpression of Nurr1. Nurr1-overexpression alone was insufficient to activate TH expression in C17.2 neural stem cell lines established from the mouse cerebellum, but soluble factors secreted by type I astrocytes were required in addition to Nurr1 (Wagner *et al.* 1999). This study was in contrast to work by Gage and colleagues who reported that Nurr1 readily induces TH expression in undifferentiated adult hippocampal precursor cells but cannot induce them to differentiate into DA neurons (Sakurada *et al.* 1999). The present study demonstrates that Nurr1-overexpression suffices for the generation of functional DA neurons from CNS precursor cells. These findings are related to the cell source of origin. In contrast to both previous studies we used short-term expanded primary CNS precursor cells that may have a distinct potential for that of established cell lines (Wagner *et al.* 1999) or long-term expanded adult neural precursors (Sakurada

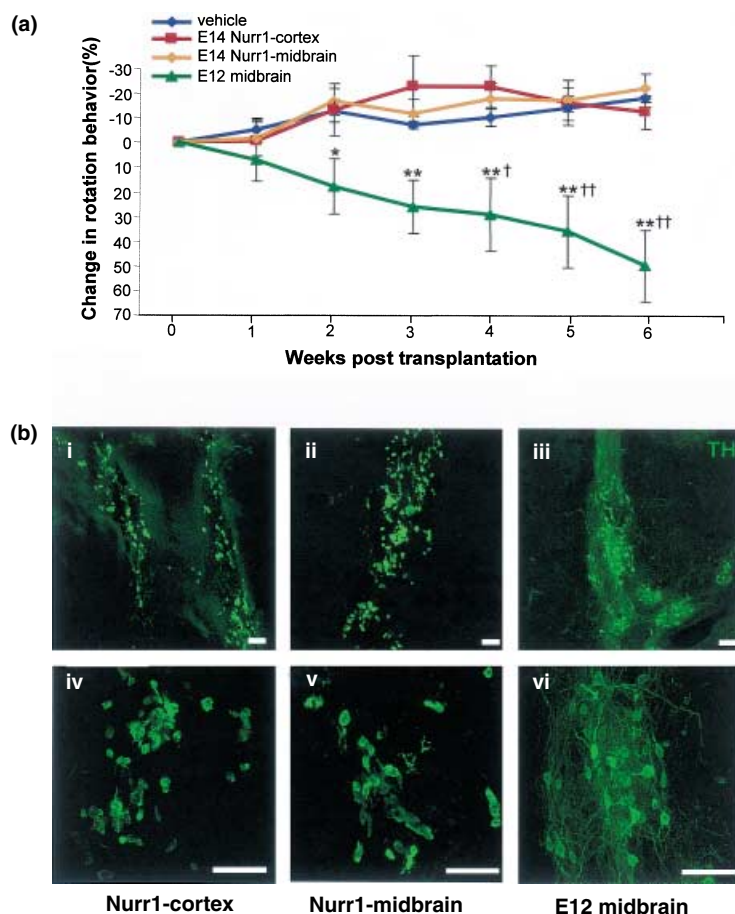


Fig. 6 (a) Time course of apomorphine-induced rotation response. Rotation scores were evaluated at 1, 2, 3, 4, 5 and 6 weeks of post-transplantation. Data are given as changes in rotation scores for each animal as compared to pre-transplantation values. None of animals grafted with vehicle (normal saline injected) control ($n = 5$), Nurr1-transduced cortical ($n = 8$) and Nurr1-transduced midbrain precursors ($n = 7$) showed significant changes of the rotation scores. In contrast, significant decrease in the rotation scores was observed in the rats grafted with E12 midbrain precursors at all time points after transplantation ($n = 7$). Data are presented as mean \pm SEM. (b) Confocal microscopic images for TH-stained sections of hemiparkinsonian rats grafted with Nurr1-induced cortical (i and iv), midbrain (ii and v) and intact E12 midbrain DA cells (iii and vi). †Significantly different from vehicle injected group. *Significantly different from Nurr1-cortical DA group. †, $p < 0.05$; ††, $p < 0.01$. Scale bar, 40 μ m.

et al. 1999). Similar to our study, it was recently demonstrated that Nurr1 constitutively expressed in mouse embryonic stem cells facilitates embryonic stem cells to differentiate into DA neurons (Chung *et al.* 2002; Kim *et al.* 2002).

DA neurons from Nurr1-induced CNS precursor showed a much less mature neuronal phenotype as those from E12 mesencephalic precursors (Fig. 5c). Midbrain DA neuron commitment is thought to occur early in brain development (between E9–E11 in the rat) (Wang *et al.* 1995; Hynes *et al.* 1995a,b, 1997) whereas TH expression is acquired much later upon terminal differentiation (Saucedo-Cardenas *et al.* 1998; Witta *et al.* 2000). Thus, most of the DA neurons generated from cultured E12 neural precursors are likely to be derived from their progenitors, having already acquired a certain level of neuronal and dopaminergic commitment but retaining their ability to proliferate at least short term. The precursor status of the cells appears crucial as our transfection of Nurr1 into differentiated cells did not show any increase in TH+. This result is consistent with previous findings reporting a correlation between proliferation rate and Nurr1-mediated DA neuron conversion in C17.2 cells (Wagner *et al.* 1999). In addition, TH+ cells could be detected even in the presence of bFGF in Nurr1-transduced

culture, whereas TH+ in E12 midbrain precursor cultures generally appear only after bFGF withdrawal. In fact some of the Nurr1-transduced TH+ cells incorporated BrdU suggesting sustained precursor status (data not shown). These findings indicate that Nurr1 acts on undifferentiated precursor cells, and that Nurr1-induced TH expression preceded terminal differentiation into neurons. Thus, the sequence of DA neuron differentiation by exogenous Nurr1 *in vitro* seems to be reversed to that of *in vivo* midbrain DA neuron differentiation where TH expression occurs several days after the expression of neuronal markers. The reason for this inversion is unclear. One possible explanation could be that bFGF causes a delayed neuronal differentiation and Nurr1 is able to overcome delayed TH expression but not the delayed expression of neuronal markers. Another possibility is that the dose of Nurr1 is critical and that superphysiological levels cause premature TH induction.

However, the mechanism by which Nurr1 promotes DA neuron differentiation remains elusive. Previous work demonstrated that Nurr1 null mutant mice undergo normal midbrain formation, patterning and neuronal differentiation including the establishment of normal projections to the striatum. However, these mesencephalic precursors are

unable to express markers of DA neuron phenotype such as TH (Witta *et al.* 2000). Direct transcriptional activation via binding to the TH promoter has been proposed as a mechanism of Nurr1 action in adult hippocampal precursor cells (Sakurada *et al.* 1999; Iwawaki *et al.* 2000). In contrast, Nurr1 overexpression in DA MN9D cells resulted in mature and highly differentiated phenotypes with long neurites (Castro *et al.* 2001). Here, we found no effect of Nurr1 on precursor cell proliferation or neuronal vs. glial differentiation, and Nurr1-induced TH⁺ cells often displayed very immature morphologies (Fig. 5c). Candidate developmental genes crucial for early midbrain DA neuron development were unchanged, whereas DA neuron markers such as TH, AADC, DAT, VMAT and Ptx3 were increased. Our results support the hypothesis that Nurr1 induces the biochemical machinery of DA neurons independent of appropriate regional patterning state of the cells and without true re-specification of neuronal subtype.

Our data did not yield any functional improvements in Parkinsonian rats grafted with Nurr1-DA cells (Fig. 6). Behavioral improvement is typically correlated to the number of surviving DA neurons. However, the minimum number of surviving DA neurons for inducing behavioral improvement in rodents is approximately 100–200 (Galpern *et al.* 1996). This suggests that the number of surviving TH⁺ cells in Nurr1-DA grafted animals is unlikely to account for the lack of behavioral improvement. DA release from grafted neurons is another essential parameter (Rioux *et al.* 1991). Even though we detected significant DA release of Nurr1-DA neurons *in vitro*, we cannot rule out that these cells exhibit decreased DA release *in vivo* or lack sufficient synaptic interaction with the host brain, another important parameter for long-term graft function (Zuddas *et al.* 1991; Bjorklund 1993; Bjorklund and Lindvall 2000; Isacson *et al.* 2001). The immature morphologies of Nurr1-DA cells (Fig. 5 and Table 2) suggest that insufficient differentiation and maturation might be key factors for the negative *in vivo* results. Future studies involving DA measurements *in vivo* should address this issue. Survival and function of donor cells is also highly dependent on trophic support (Rosenblad *et al.* 1996; Zawada *et al.* 1998) and can be adversely affected by immunologic factors (Larsson *et al.* 2000). Nurr1-DA cells exhibited reduced *in vivo* survival and maturation, compared with E12 precursor-derived DA cells. Further studies should address whether inflammatory host responses or specific growth factor requirements of Nurr1-DA cells may be responsible for the lack of *in vivo* function.

Although neural stem cells are thought to provide a renewable source of specific neurons, the challenges remain to overcome limitations in long-term propagation and difficulties in specifying neuronal subtypes. The *in vitro* generation of rat DA neurons with successful *in vivo* grafting data is currently still limited to cells isolated from E11.5–E12.5 ventral midbrain (Studer *et al.* 1998; Lee *et al.* 2003).

Moreover, the efficiency of DA neuron generation from mesencephalic precursors steeply decreases after long-term expansion and passaging (Yan *et al.* 2001). The present study provides a potential strategy on how these limits could be overcome. The forced expression of Nurr1 leads to the efficient generation of DA-producing TH⁺ neurons from passaged CNS precursors regardless of embryonic stages and brain regions of origin from which precursors were isolated. Future studies will need to address the limited *in vitro* and *in vivo* maturation and DA neuron function of Nurr1-induced CNS precursors to make these cells a valid alternative to the use of midbrain precursors or primary fetal tissue. However, this study provides a first step towards a renewable CNS precursor-based generic source of DA neurons for cell therapy in Parkinson's disease.

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