

Temporally induced Nurr1 can induce a non-neuronal dopaminergic cell type in embryonic stem cell differentiation

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Abstract

The nuclear transcription factor Nurr1 is involved in the development and maintenance of the midbrain dopaminergic (DA) neuronal phenotype. We analysed the cellular and biological effects of Nurr1 during embryonic stem (ES) cell differentiation using the ROSA26-engineered Tet-inducible ES cell line J1-rtTA that does not express transgenes in mature neurons. Induction of Nurr1 at nestin-positive precursor and later stages of ES cell differentiation produced a non-neuronal DA cell type including functional DA transporters. In these cells, we found a clear correlation between Nurr1 and TH gene expression and specific midbrain DA cellular markers such as AADC, AHD2 and calbindin. Nurr1 did not alter gene expression of non-DA neuronal phenotypes and did not influence other midbrain developmental transcription factors, such as Otx1, Otx2, En-1, GBX2, Pitx3 and *lmx1b*. In addition, Nurr1 expression was required for maintenance of the DA phenotype and mediated up-regulation of the tyrosine kinase Ret and associated trophic factor GDNF-family receptors α 1, 2, and 4. This demonstrates that Nurr1 is sufficient to induce and maintain a midbrain-like DA biochemical and functional cellular phenotype independent of neurogenesis.

Introduction

Nurr1 has been proposed as the determinant factor in the phenotypic cellular specification of midbrain DA neurons *in vivo* (Zetterstrom *et al.*, 1997; Castillo *et al.*, 1998; Saucedo-Cardenas *et al.*, 1998). *In vitro*, over-expression of Nurr1 in ES cells facilitates the generation of DA neurons (Chung *et al.*, 2002; Kim *et al.*, 2002) and in neural precursors, Nurr1 activates tyrosine hydroxylase (TH) expression (Sakurada *et al.*, 1999) or, perhaps, stimulates generation of DA producing neurons (Wagner *et al.*, 1999; Kim *et al.*, 2003a). It can directly activate the TH promoter (Sakurada *et al.*, 1999; Iwawaki *et al.*, 2000; Kim *et al.*, 2003b) and has also been implicated in the transcriptional activation of the dopamine transporter (DAT) gene (Sacchetti *et al.*, 2001). These data suggest that Nurr1 plays a pivotal role in specifying a DA phenotype in the neuronal context, but its role in directing a neuronal midbrain-specific phenotype is still unclear. Thus, some investigators claim a role of Nurr1 in neurogenesis *in vivo* (Zetterstrom *et al.*, 1997) or neuronal differentiation *in vitro* (Kim *et al.*, 2003c), whereas others suggest that Nurr1 is not necessary for ventral mesencephalon (VM) neurogenesis (Saucedo-Cardenas *et al.*, 1998; Wallen *et al.*, 1999; Witta *et al.*, 2000; Kim *et al.*, 2003a). These data imply, that there are two potentially different characteristics of this transcriptional activator: (i) cell fate determination and (ii) specification and/or maintenance of a biochemical identity. Commonly it is thought that both aspects are tightly linked and depend on each other,

e.g. Nurr1 specifies the DA phenotype in the context of neurogenesis. Another possibility, however, could be that the DA-specific biochemical cell type is controlled by a genetic subprogram dominated by Nurr1 activation that does not require a neuronal context. This possibility can be addressed by cell type specific expression and by inducibly expressing Nurr1 during different stages of ES cell differentiation.

ES cells are pluripotent cells, which can be maintained *in vitro* as immature cells, or differentiated into specific cell types, thus providing tools for analysing cell development (Hooper *et al.*, 1987; Nagy *et al.*, 1993; Dinsmore *et al.*, 1996) and as a potential source for cell therapy (Weissman, 2000; Bjorklund *et al.*, 2002; Daley, 2002; Kim *et al.*, 2002; Le Belle & Svendsen, 2002; Rossi & Cattaneo, 2002). ES cells can also be genetically engineered to express foreign molecules constitutively. Temporally controlled expression of exogenous genes, however, is desirable because cell fate-determining factors are usually expressed in a stage-dependent manner. This can be achieved with inducible gene expression systems, such as the tetra- or doxycycline-dependent Tet-system (Gossen & Bujard, 1992; Gossen *et al.*, 1993), which contains two expression units for a transactivator (TA) binding to doxycycline and a Tet-response element (TRE) promoter to drive the expression of the desired genetic material. To achieve inducible gene expression in ES cells, the gene of the rtTA (Gossen *et al.*, 1995) has recently been inserted into the mouse ES cell line J1 (Wutz & Jaenisch, 2000).

Here, we have analysed J1-rtTA ES cells *in vitro* for their capability of neurogenesis and to inducibly express transgenes during cell development using humanized renilla green fluorescent protein (hrGFP) gene expression. J1-rtTA-hrGFP cells expressed the transgene in immature ES cells, neural precursors, in 5–10% of astrocytes, but

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not in mature neurons. This provided us with the opportunity to study the biological effects of induced Nurr1 expression in ES cell differentiation independent of its neuronal identity. Our studies show that induction of Nurr1 at later stages of ES cell differentiation led to the development of a full DA phenotype in non-neuronal cell populations.

Materials and methods

Plasmid construction and generation of recombinant cell lines

To generate recombinant Tet-response promoter-containing pTRE2 plasmids, a PmeI–XbaI mouse Nurr1 cDNA fragment was inserted into the EcoRV and XbaI sites and a SacII–SalI hrGFP minigene into the corresponding sites of the pTRE2 vector (Clontech, Palo Alto, CA). The insertion of the cDNAs into the vectors was confirmed by restriction digestion and sequence analysis. J1-rtTA ES cells [kindly provided by Dr R. Jaenisch (Wutz & Jaenisch, 2000)] were cotransfected with 30 µg of the pTRE2-Nurr1 or pTRE-hrGFP vectors, and 3 µg of the puromycin resistant gene containing plasmid pPGK-puro (Clontech) by electroporation using a Bio-Rad GenePulser set at 0.28 µF and 500 V. Stably transfected cells were selected in ES cell medium containing 2 µg/mL puromycin (Sigma, St Louis, MO). Puromycin-resistant clones were isolated and expanded in the absence or presence of 1 µg/mL doxycycline (Sigma) and tested for inducible transgene expression either by fluorescence microscopy (GFP) or RT-PCR (Nurr1) as described below. Two regulatory hrGFP and seven Nurr1-expressing J1-rtTA clones were selected and maintained in ES cell medium containing 500 µg/mL G418 (Clontech) and 2 µg/mL puromycin (Sigma). The two hrGFP clones and five out of the seven regulatory J1-rtTA-Nurr1 clones were further analysed in *in vitro* differentiation experiments.

ES cell culture and *in vitro* differentiation

Recombinant J1-rtTA cells were propagated and differentiated *in vitro* as described previously (Lee *et al.*, 2000; Chung *et al.*, 2002). Transgene expression was induced by adding 1 µg/mL doxycycline (Sigma) to the tissue cultures, according to an induction protocol as outlined in Fig. 4A. Induced GFP expression was measured at different stages of cell differentiation by fluorescence microscopy and Nurr1 expression by RT-PCR.

Immunocytochemistry

Cells were analysed by immunofluorescence staining as previously described (Chung *et al.*, 2002) and examined using a Leica TCS/NT confocal microscope equipped with krypton, krypton/argon and helium lasers. The following primary antibodies were used: mouse anti-beta-tubulin (Covance, Richmond, CA; 1 : 500), rabbit anti-beta-tubulin (Covance; 1 : 2000), sheep antityrosine hydroxylase; TH (Pel-Freez, Rogers, Arkansas; 1 : 300), rabbit anti-TH (Pel-Freez; 1 : 300), mouse anti-TH (Pel Freez; 1 : 300), rabbit anticholine acetyltransferase; ChAT (Chemicon International, Temecula, CA; 1 : 750), rabbit antigamma aminobutyric acid; GABA (Sigma, St Louis, MI; 1 : 5000), rabbit antiglutamate; Glu (Sigma; 1 : 10 000), rabbit antiglial fibrillary acidic protein; GFAP (DAKO, Carinteria, CA; 1 : 500), mouse anti-Nestin (Developmental Studies Hybridoma Bank, Iowa City, IO; 1 µg/mL), mouse anti-SSEA1 (Dev. Studies Hybridoma Bank; 1 µg/mL), rat antidopamine transporter; DAT (Chemicon Intl.; 1 : 1000), sheep antiaromatic L-amino acid decarboxylase; AADC (Chemicon; 1 : 200), goat anti-Ret (Santa Cruz, Santa Cruz, CA; 1 : 50), mouse anti-NeuN (Chemicon; 1 : 100), mouse anti-O4 (Chemicon; 10 µg/mL), mouse anti-GalC (Chemicon; 1 µg/mL), goat antivesicle monoamin transporter 1; VMAT1 (Santa Cruz; 4 µg/mL), rabbit anti-VMAT2

(Pel-Freez; 1 : 300), sheep antiphenylethanolamine-N-methyltransferase; PNMT (Chemicon, 1 : 100), rat antiembryonic neural cell adhesion molecule (CD56); PSANCAM (BD Pharmingen, San Diego, CA (1 : 250), rabbit anti-Cytokeratin (Dako; 1 : 400), mouse anti-Desmin (Dako, 1 : 50). Fluorescent-labelled secondary antibodies were Cy2-, Rhodamine Red-X-, or Cy5-labelled donkey IgG (Jackson Immunoresearch Laboratory).

RNA preparation and semiquantitative RT-PCR

Total RNA from plated cells at different stages in the differentiation protocol was prepared using TriReagent (Sigma) followed by treatment with DNase I (DNA-free, Ambion). For RT-PCR analysis, 3–5 µg RNA was transcribed into cDNA with the SuperScript™ Pre-amplification Kit (Life Technologies) and oligo (dT) primers. The cDNA was then diluted 1 : 3 and 2.5% per reaction were analysed in a PCR assay using the following mouse-specific primers.

β-actin:

5'-GGTGATGACCTGGCCGTCAGGCAGCTCGTA-3';
5'-AACCCCAAGCCAACCGCGAGAAGATGACC-3' (402 bp).

rtTA:

5'-CAATTACGGGTCTACCATCG-3';
5'-TCTGCTCAAACCTCGAAGTCG-3' (319 bp).

hrGFP:

5'-CTGCAGGAGATCATGAGCTTC-3';
5'-GGTACACGAACATCTCCTCG-3' (339 bp).

Nurr1:

5'-CATGGACCTCACCAACACTG-3';
5'-GAGACAGGTGTCTTCTCTG-3' (383 bp).

TH:

5'-TCCTGCACTCCCTGTGAGAG-3';
5'-CCAAGAGCAGCCCATCAAAGG-3' (423 bp).

AADC:

5'-CCTACTGGCTGCTCGGACTAA-3';
5'-GCGTACCAGGACTCAAACCTC-3' (715 bp).

DAT:

5'-CAGAGAGGTGGAGCTCATC-3';
5'-GGCAGATCTTCCAGACACC-3' (328 bp).

Pitx3:

5'-CTCTCTGAAGAAGAAGCAGCG-3';
5'-CGCCCAGGCCCTGCAAGGCTC-3' (491 bp).

AHD2:

5'-CTGCAAGTGAGGAGGTCATC-3';
5'-CTGCTGGCTTGACAACCAC-3' (458 bp).

Calbindin:

5'-GCAGTCATCTCTGATCACAGC-3';
5'-GAGGTCTGTGTACTCTGCTAG-3' (428 bp).

DBH:

5'-CTGGACAGGCATAAATGGCAG-3';
5'-GTCTTGCTGGGAATCCAGATG-3' (449 bp).

GluT:

5'-AAAGTTCAGAGCCTCACCAAG-3';
5'-GATCAAATCCAGGAAGGCATC-3' (429 bp).

TPH:

5'-CTACACTCCAGAGCCAGACAC-3';
5'-GACATCAAGGTCATACCGCAAC-3' (501 bp).

GBX2:

5'-GAGCATCACACAGGGTTCTG-3';
5'-CACCTTTAAATCGCGCTCCTC-3' (370 bp).

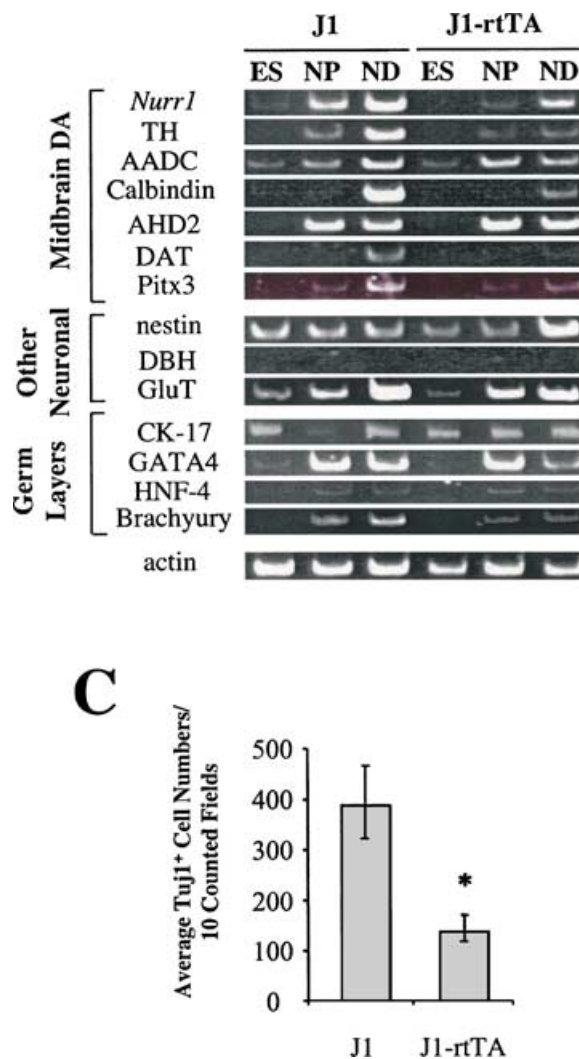
Brachyury:

5'-ACAATTCATCTGCTTGTCTGTCC-3';
5'-CGGTTGTTACAAGTCTCAGCAC-3' (436 bp).

HNF-4:

5'-GAGGTCCATGGTGTTTAAGGAC-3';
 5'-CTGCAGCAGGTTGTCAATCTTGG-3' (410 bp).
 CK-17:
 5'-GAAGAACAAGATCCTTGTGGCC-3';
 5'-CTTGCTGAAGAACCAGTCTTCG-3' (411 bp).
 GATA4:
 5'-AGATGCGCCCCATCAAGACAG-3';
 5'-CCGGAACACCCATATCCTAAG-3' (413 bp).
 Ret:
 5'-TCCCAGAGTGAGTTACGAGACCTG-3';
 5'-GACAGCCCCAAGTCGGAAAT-3' (395 bp).
 GFR α 1:
 5'-CTTTGAGTGGCCAGAGGAGC-3';
 5'-TGTGTTCCACTTGGCTGGAAAAC-3' (559 bp).

The primer sequences for amplifying the following genes were previously published: Otx1, Otx2, En-1 and Nestin (Lee *et al.*, 2000); GFR α 2 (Wong & Too, 1998); GFR α 4 (Lindahl *et al.*, 2000).



PCR reactions were carried out with $1 \times$ IN reaction buffer (Epicentre Technologies, Madison, WI), 1.4 nM of each primer, and 2.5 units of Taq I DNA polymerase (Promega, Madison, WI). Samples were amplified in an Eppendorf Thermocycler (Brinkmann Instruments, Westbury, NY) under the following conditions: denaturing step at 95 °C, 40 s; annealing step at 60 °C, 30 s; amplification step at 72 °C, 1 min for 25–35 cycles and a final amplification step at 72 °C, 10 min. For semiquantitative PCR, cDNA templates were normalized by amplifying actin-specific transcripts and levels of gene transcription were detected by adjusting PCR cycling and primer design in such a way that each primer set amplified its corresponding gene product at its detection threshold to avoid saturation effects. Twenty to 40% of the PCR products were analysed in 7% polyacrylamide gels. Gels were then stained with ethidium bromide and visualized under UV light and photographed on Polaroid 3000 black and white prints. Photographs were scanned with an Epson (Epson Perfection 1640SU) scanner.

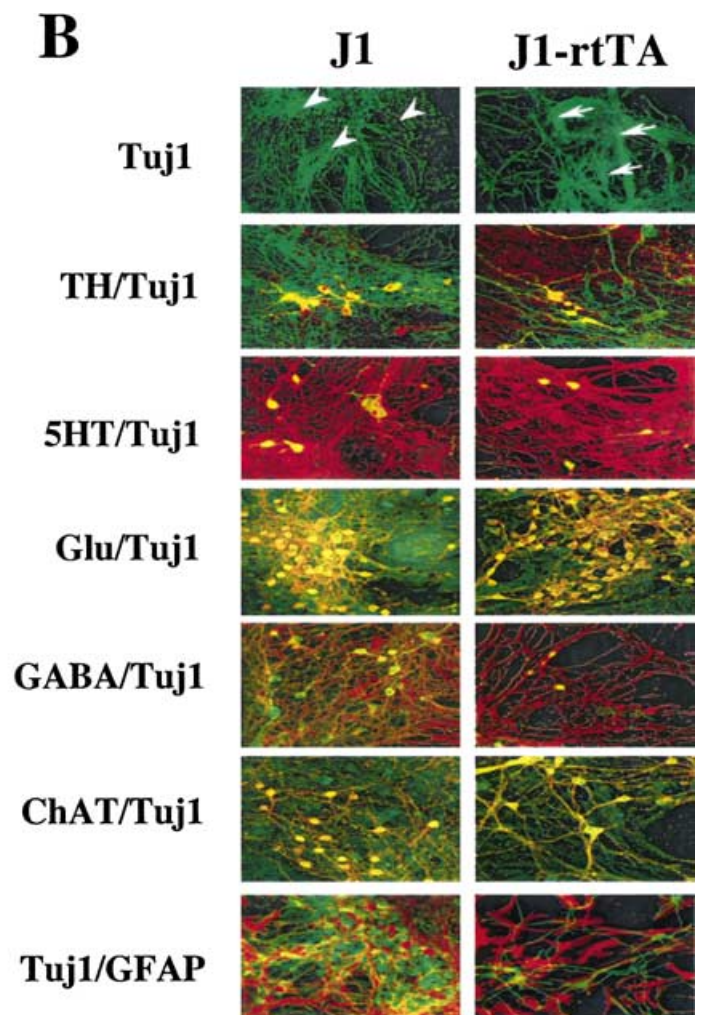


Fig. 1. Characterization of J1 and J1-rtTA ES cells in *in vitro* differentiation. (A) Midbrain DA, other neuronal and germ layer marker gene expression during differentiation of J1 and J1-rtTA cells. ES cells were *in vitro* differentiated as described (Lee *et al.*, 2000; Chung *et al.*, 2002) and gene expression was analysed by RT-PCR. Stages of cell development (Lee *et al.*, 2000; Chung *et al.*, 2002) are indicated as follows: ES, embryonic stem cells; NP, neural precursors; ND, neural differentiation. (B) Neural phenotypes after *in vitro* differentiation of naïve J1 and J1-rtTA ES cells. Images of single-stained Tuj1 (green) and double-stained for TH, 5HT, Glu, GABA, ChAT, or GFAP (red) and Tuj1 (green). Double-stained cells appear yellow. Note that neurons derived from naïve J1 ES cells develop into large clusters of neurons with short processes (arrow heads in upper row), whereas the J1-rtTA-derived neurons aggregated in smaller clusters and developed bundles of long neurites (arrows in upper row). (C) Cell counts of Tuj1⁺ neurons after differentiation of naïve J1 and J1-rtTA. Shown is the summary of three individual experiments performed. * $P < 0.0001$, with ANOVA and Fisher's PLSD posthoc analysis.

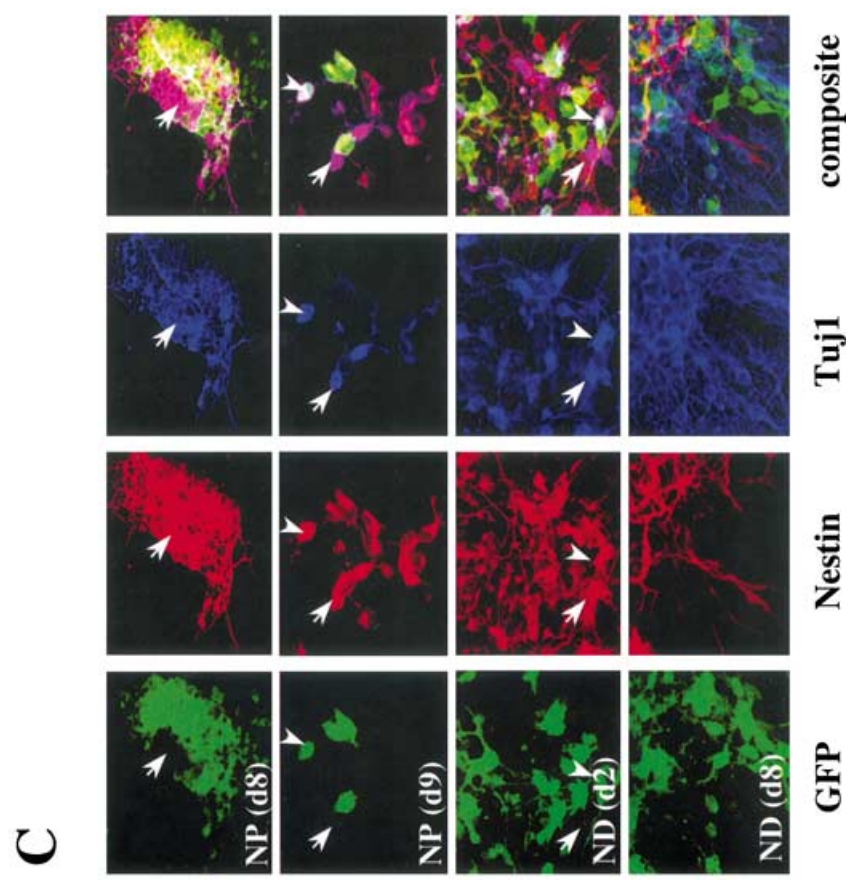
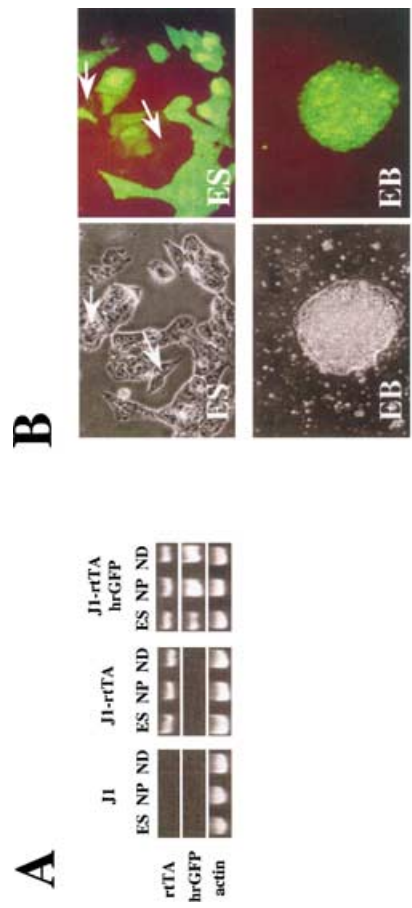
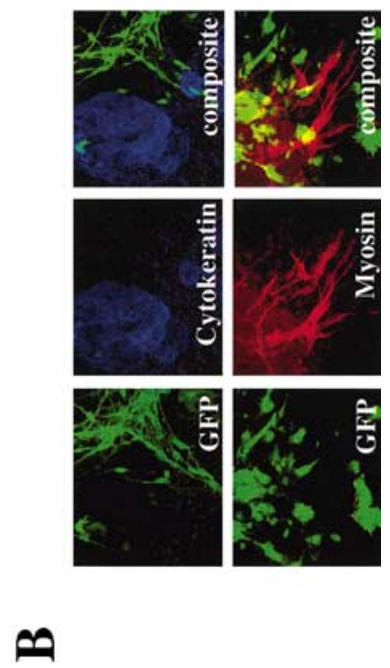
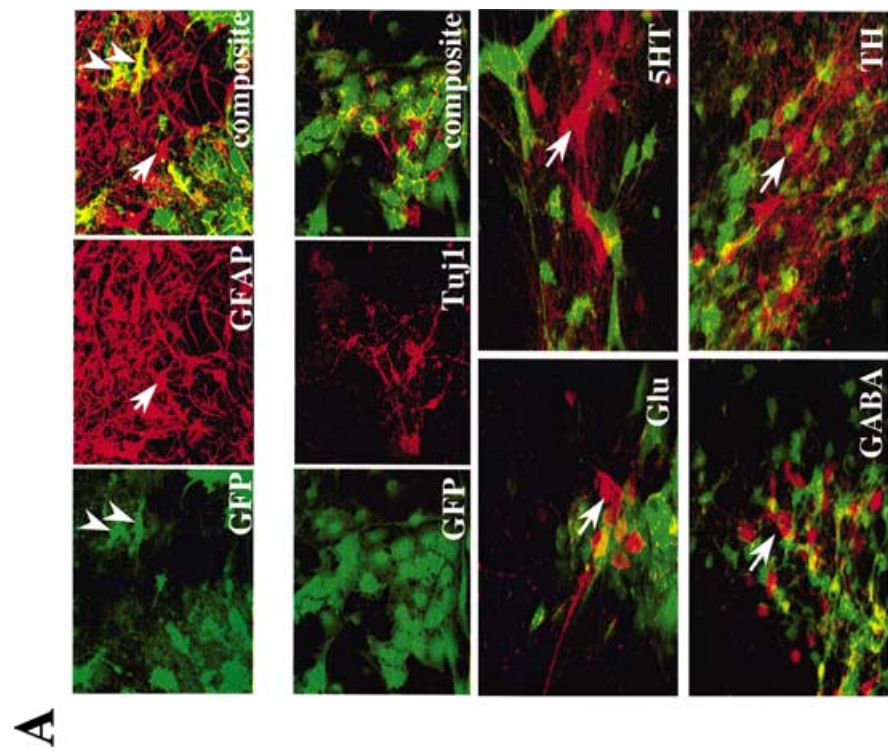


Fig. 3.

Fig. 2.

The optical densities (OD) of PCR signals were measured using the NIH Image software (National Institutes of Health, USA), version 1.61. After subtracting background signals, the intensity of each individual signal was calculated over its corresponding actin signal and plotted in relative units of OD.

[³H]Dopamine uptake assay

The uptake of [2,5,6-³H]dopamine (specific activity 34.2 mCi/mg, Amersham Pharmacia Biotech., Buckinghamshire, England) was conducted at 37 °C in a CO₂ incubator. Cells were differentiated in 24-well plates and incubated at stage 5 day 15 for 20 min with or without 1 μM of the DAT inhibitor GBR 12909 (Sigma). [³H]dopamine (25 nM) was then added and incubation continued for 20 min. The incubation medium was removed and the cells were washed three times with ice-cold PBS, dissolved in 0.5 M NaOH and neutralized with HCl. Aliquots were taken for scintillation counting and protein determination. Specific binding was calculated by subtracting the uptake values obtained in the presence of GBR-12909.

Cell counting and statistical analyses

Numbers of TH⁺ and Tuj1⁺ cells were counted per field at 63 × magnification using a Zeiss Axioplan I fluorescent microscope and performed on blind coded samples (cultures grown on coverslips) by two independent investigators per sample. Ten fields were randomly selected and counted for each sample. When not stated differently, numbers in figures represent the average of Tuj1⁺, TH⁺/Tuj1⁺, or TH⁺/Tuj1⁻ cells from three to five samples per assay. For statistical analysis, the Statview software was used for performing analysis of variance (ANOVA) with an α -value of 0.01 to determine possible statistical differences between group means. When significant differences were found, post hoc analysis was performed using Fisher's PLSD ($\alpha = 0.05$).

Results

Neurogenesis and expression of marker genes during in vitro differentiation of J1 and J1-rtTA ES cells

To regulate transgene expression, we used the ES cell line J1-rtTA (Wutz & Jaenisch, 2000). To determine whether gene-engineering ES cells at the ROSA26 locus affected the cell's differentiation capacities, we performed comparative *in vitro* differentiation experiments using naïve J1 or recombinant J1-rtTA ES cells. Cells were differentiated according to previously published protocols (Lee *et al.*, 2000; Chung *et al.*, 2002). In both cell lines, RT-PCR analyses at different stages of cell development revealed similar patterns, but different levels of gene expression for the neuron precursor marker Nestin, and for neuronal markers such as glutamate transporter (GluT) and the midbrain DA markers Nurr1, Pitx3, TH, aromatic L- amino acid decarboxylase (AADC), aldehyde-dehydrogenase (AHD2), calbindin and DAT

(Fig. 1A). These results are consistent with a reduced overall neurogenesis (Fig. 1C, see below) and consequently also relative lower amounts of TH⁺ neurons (data not shown) in the J1-rtTA ES cell line. In addition, the noradrenergic marker decarboxyhydroxylase (DBH) was not expressed in mature neurons developed under these culture conditions (Chung *et al.*, 2002). Both cell lines also showed no differences in the expression patterns of germ layer markers as analysed by the ectodermal marker Cytokeratin 17 (CK-17) (McGowan & Coulombe, 1998), the early endodermal transcription factor GATA4 (Soudais *et al.*, 1995), the late endodermal factor hepatic nuclear factor 4 (HNF4) (Li *et al.*, 2000) and the mesodermal marker Brachyury (Wilkinson *et al.*, 1990).

To evaluate neurogenesis, *in vitro* differentiated mature J1 and J1-rtTA ES cells were analysed by immunocytochemistry (ICC). Both cell lines developed into Tuj1⁺ neurons including dopaminergic (TH⁺), serotonergic (5HT⁺), glutamergic (Glu⁺), GABA-ergic (GABA⁺) and cholinergic (ChAT⁺) subtypes as well as GFAP⁺ astrocytes (Fig. 1B). Whereas the naïve J1 ES cells grew into large clusters of neurons with short processes (Fig. 1B, upper row, arrow heads), the J1-rtTA-derived neurons aggregated in smaller clusters and developed bundles of long neurites (Fig. 1B, upper row, arrows). In addition, quantification of neurons after differentiation revealed an ~60% reduction in neurogenesis in J1-rtTA ES cell development when compared to the naïve J1 cell line (Fig. 1C). Taken together, these results demonstrate that ROSA26-engineered J1-rtTA cells have reduced neurogenesis compared to the parental J1 ES cell line.

Induced transgene expression in various cell types after differentiation of J1-rtTA ES cells

To test inducible transgene expression in J1-rtTA ES cells, we generated stable transfected J1-rtTA-hrGFP cell clones and analysed them in *in vitro* differentiation experiments. RT-PCR analyses showed that in the parental J1-rtTA and the transgenic J1-rtTA-hrGFP cells, the rtTA gene was constitutively expressed at all stages (ES, NP, and ND) of cell development (Fig. 2A). In addition, doxycycline-dependent hrGFP transcription could be up-regulated within 3–6 h and protein expression was visualized by fluorescence microscopy 12 h after gene induction indicating functional regulatory gene expression (data not shown). When ES cells were analysed for induced GFP expression, >90% of the cells were GFP-positive, whereas ~5% did not express the transgene (Fig. 2B, upper row). During differentiation, all EBs were GFP positive (Fig. 2B, lower row), but the transgene was only partly expressed at the NP cell stage (Fig. 2C). When analysed by ICC, GFP was expressed in Nestin⁺/Tuj1⁻ and Nestin⁺/Tuj1⁺ precursors at late stages of NP (day 8 and 9). However, ~50% of the Nestin⁺/Tuj1⁺ precursors were GFP-negative (Fig. 2C, upper two rows). At early stages of ND (day 1–8), immature Nestin⁺/Tuj1⁺ neurons continued to partially express GFP (Fig. 2C, lower middle row), but became GFP-negative, when differentiating into Nestin⁻/Tuj1⁺ mature neurons

Fig. 2. hrGFP expression in differentiating J1-rtTA-hrGFP cells. Cells were *in vitro* differentiated and hrGFP gene expression was induced by adding 1 μg/mL doxycycline to the tissue cultures. (A) RT-PCR detecting rtTA and hrGFP gene expression at different time points of *in vitro* differentiation. (B) Brightfield (left) and fluorescence (right) images of undifferentiated ES cells and EBs. Arrows show GFP negative cells. (C) hrGFP expression in NPs and immature neurons during differentiation of J1-rtTA-hrGFP cells. ICC images for Nestin (red) and Tuj1 (green) on NPs prior (day 8, upper row) and after (day 9, upper middle row) plating for ND. Lower middle and lower rows show Nestin- and Tuj1-stained cells during ND at day 2 and day 8, respectively. The right column shows overlap images. Arrows indicate cells, which are GFP negative and arrowheads denote cells expressing the GFP transgene. Note that some immature neurons (Nestin⁺/Tuj1⁺) in NP and at day 2 of ND express the GFP gene, while mature neurons at day 8 of ND (Nestin⁻/Tuj1⁺) are GFP negative.

Fig. 3. hrGFP expression in mature neural cell populations. (A) ICC images for GFAP (red, upper row), Tuj1 (red, middle row) and Glu, GABA, 5HT, and TH (red, lower rows) in fully differentiated J1-rtTA-hrGFP cells. Arrows indicate cells, which are GFP negative and arrowheads denote cells expressing the GFP transgene. Note that some of the astrocytes (GFAP⁺) express GFP, while all neuronal subtypes lack transgene expression. (B) Induced GFP expression in non-neuronal cells after differentiation: Cytokeratine (blue) and Myosin (red).

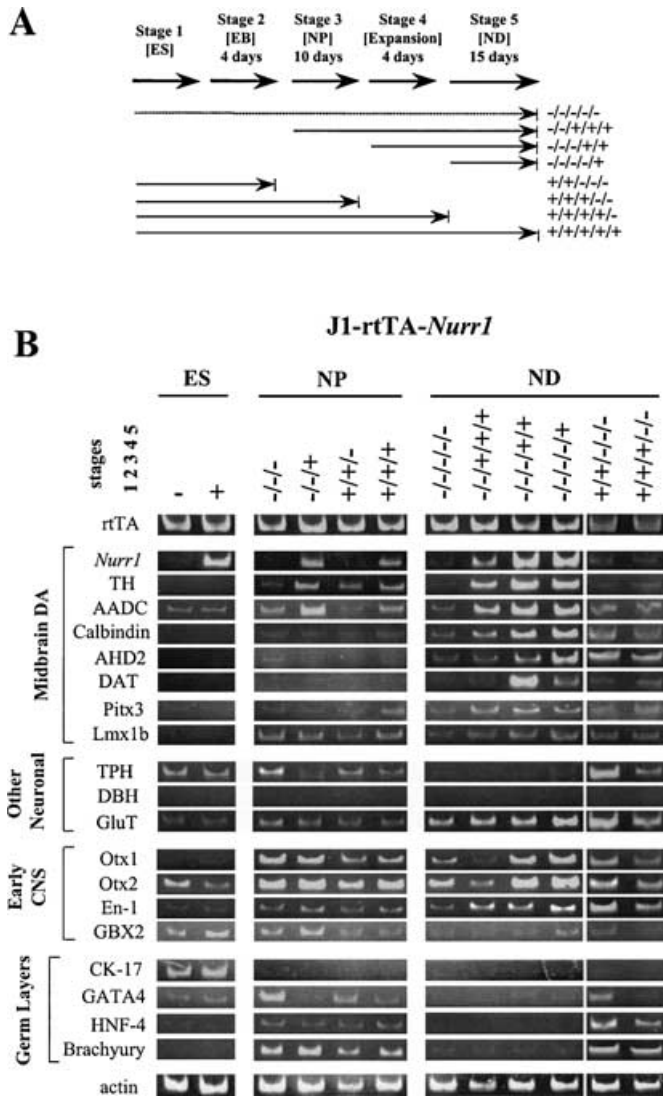


Fig. 4. Gene expression profile and neurogenesis of *in vitro* differentiated J1-rtTA-Nurr1 ES cells. (A) Scheme of inducible Nurr1 expression during *in vitro* differentiation. Stages of cell development are as described in legend of Fig. 1. For the induction of Nurr1 expression, cells were cultured in the presence (+) or absence (-) of doxycycline during the different stages of the protocol (indicated by arrows) and were analysed at the ES cell stage, at the end of stage 3 (NP) and/or at the end of the protocol (stage 5, ND). (B) Midbrain DA, other neuronal, early CNS, and germ layer marker gene expression in J1-rtTA-Nurr1 cells at different stages (ES, NP, ND) during differentiation. Nurr1 expression was induced as schematically shown in A, and gene expression was analysed by RT-PCR.

(Fig. 2C, lower row). At later stages of ND (>day 8), fully differentiated cells lacked GFP expression in mature Tuj1⁺ neurons, including neuronal subtypes such as TH⁺, 5HT⁺, GABA⁺, and Glu⁺ neurons (Fig. 3A). In contrast, GFP was expressed in a fraction of GFAP⁺ astrocytes. These data indicate that GFP expression was partially induced in Nestin⁺ precursors and immature (Nestin⁺) neurons, while loss of GFP expression occurred in mature (Nestin⁻) neurons. To further characterize the GFP⁺ cell populations, we performed additional ICC using germ layer-specific antibodies. GFP⁺ cells did not express ectodermal (Cytokeratin) or mesodermal markers, such as Myosin, Brachyury and Desmin (Fig. 3B and unpublished data). In conclusion, inducible transgene expression is effective in recombinant J1-rtTA ES cells during all stages of ES cell differentiation but does not occur in mature neurons.

Nurr1 induces DA marker genes in differentiated non-neuronal (Tuj1⁻) J1-rtTA cells

The lack of transgene expression in neurons derived from recombinant J1-rtTA ES cells provided an opportunity to study the functional properties of the 'neuronal' transcriptional activator Nurr1, and its ability to determine neuronal cell fate and/or DA specification during cell development. We generated J1-rtTA-Nurr1 ES cell clones and *in vitro* differentiated five selected clones using the induction conditions as outlined in Fig. 4A. As all clones behaved similarly in the differentiation protocols, the results of two representative clones are presented in this study.

Using semiquantitative RT-PCR at different stages of cell differentiation (Figs 4B and 5A), we found a clear correlation between Nurr1 and TH gene expression in NP and ND cell stages and a significant increase of other midbrain DA specific markers such as AADC, AHD2, calbindin and DAT, but not Pitx3 and lmx1b, when Nurr1 was induced starting from stage 3 (-/-/+ / +/+), stage 4 (-/-/+ / +/+) and stage 5 (-/-/+ / +/+). In contrast, the expression of these marker genes was not enhanced when Nurr1 induction was started at early stages (1 and 2) of differentiation. Induced expression of Nurr1 did not alter gene expression of other neuronal phenotypes such as Glu (GluT), noradrenergic (DBH) or serotonergic (tryptophane hydroxylase, TPH) neurons (Fig. 4B).

We also analysed the effects of Nurr1 on gene expression of transcription factors, such as Otx1, Otx2, engrailed-1 (En-1) and GBX2, which have been shown to play a role within the midbrain-hindbrain territory and ventral mesencephalic development at early embryonic stages (Rhinn & Brand, 2001). Induced Nurr1 did not influence the overall expression pattern of these genes during ES cell differentiation (Fig. 4B).

As our *in vitro* differentiation protocol included the generation of EBs, we additionally addressed the question whether Nurr1 had an effect on germ layer formation. We found that transcription of CK-17 was not influenced by induced Nurr1 expression. Expression of

Fig. 5. Gene expression profile and neurogenesis of *in vitro* differentiated J1-rtTA-Nurr1 ES cells. (A) Quantitative analysis of gene expression of the midbrain DA markers shown in Fig. 4B for two independently differentiated J1-rtTA-Nurr1 clones (#29 and #32). The optical densities (ODs) of PCR signals were measured and relative units were calculated over corresponding actin signals (see Materials and methods for details). (B) Cell counts of Tuj1⁺ neurons after differentiation of naive J1-rtTA and J1-rtTA-Nurr1 ES cells in non-induced and stage 3 (-/-/+ / +/+) gene induction conditions. Shown is the summary of cell counts from three individual experiments performed on two to four individual clones. **P* < 0.05, with ANOVA and Fisher's PLSD posthoc analysis. (C) Neuronal phenotypes after *in vitro* differentiation. Overlap images of double-stained cells for Glu, GABA, ChAT (red) and Tuj1 (green) or single stained for GFAP (red) from naive J1-rtTA and stage 3 (-/-/+ / +/+) Nurr1-induced J1-rtTA cells.

Fig. 6. Development and characterization of TH⁺ cell populations after *in vitro* differentiation. (A) ICC for TH (red) and Tuj1 (green) after differentiation of non-induced (-/-/-/-/-) and stage 4 (-/-/+ / +/+) Nurr1-induced J1-rtTA ES cells. Upper row: TH⁺/Tuj1⁺ neurons (white arrow-heads). Lower row: TH⁺/Tuj1⁻ cells developed during stage 4 Nurr1 induction condition. (B) Co-expression of TH (green), NeuN (red) and Tuj1 (blue) in stage 4-induced cells. (C and B) TH⁺/Tuj1⁻ cells do not express markers for neural precursors or stem cells. ICC for TH (red), Tuj1 (green), Nestin (blue in C) or SSEA1 (green in D and white arrow-heads) expression in differentiated cells after stage 3 Nurr1 induction.

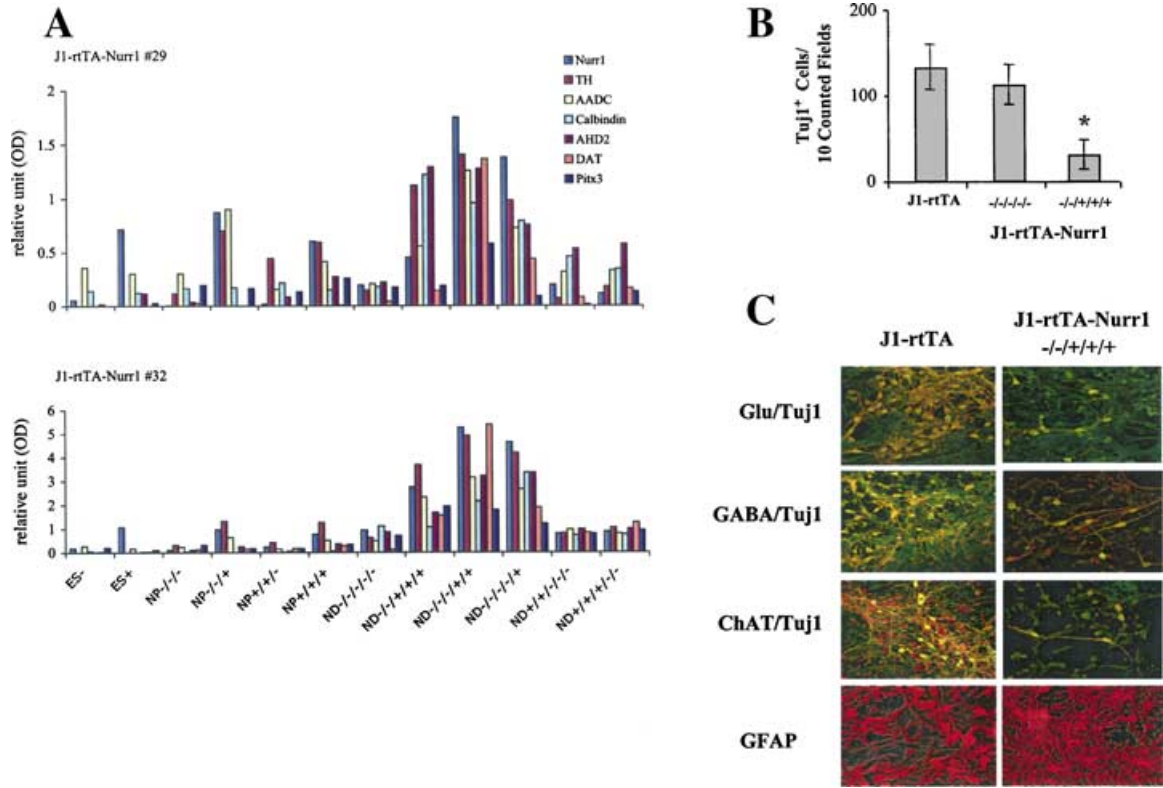


FIG. 5.

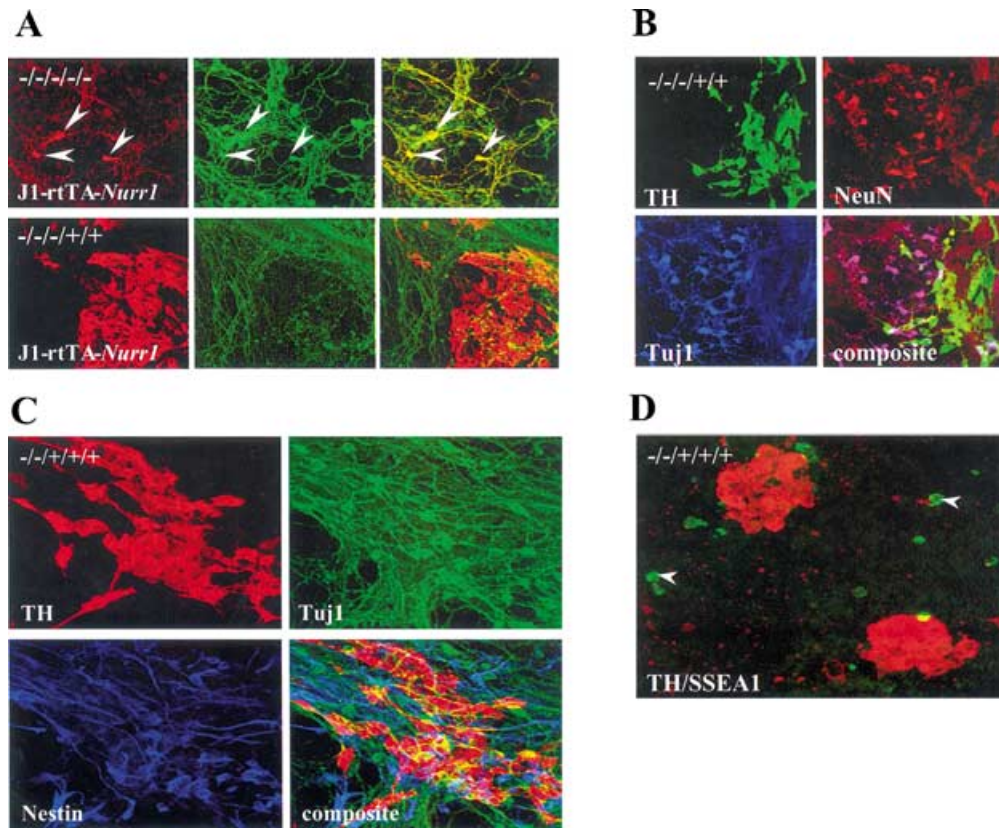


FIG. 6.

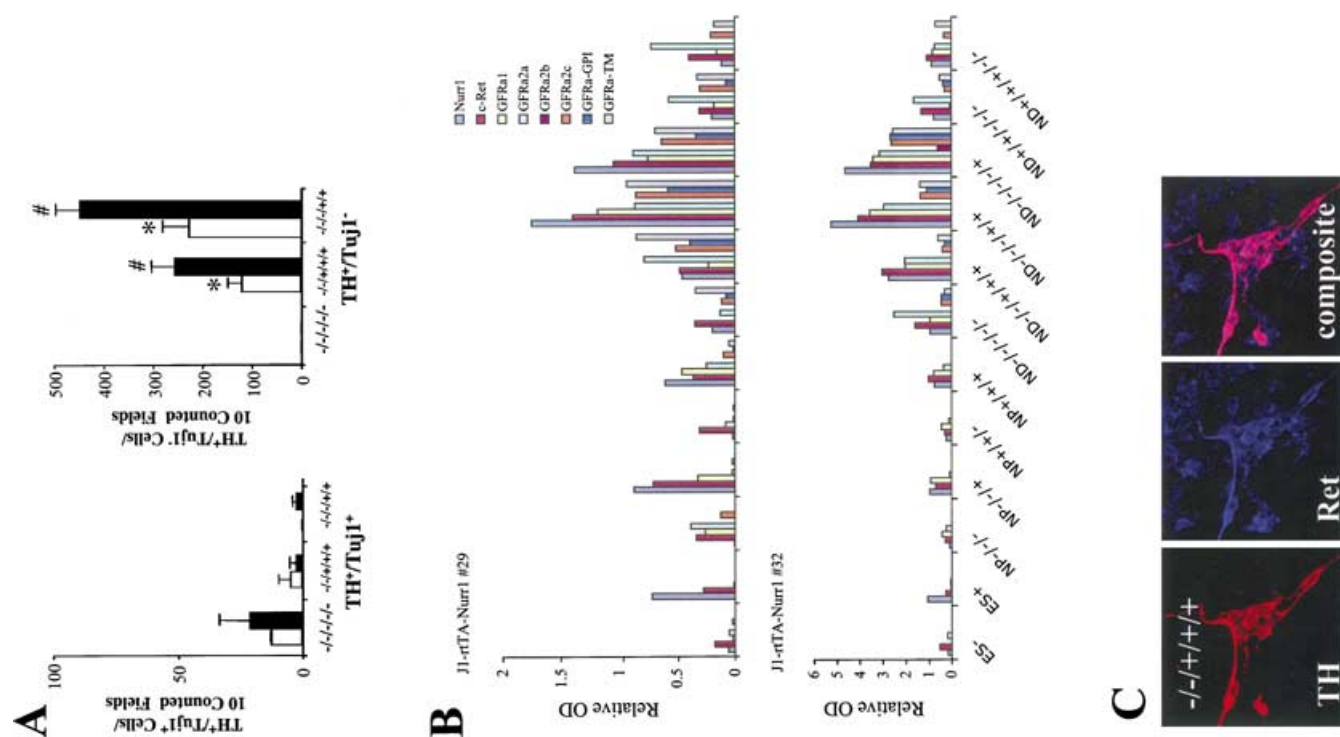


FIG. 8.

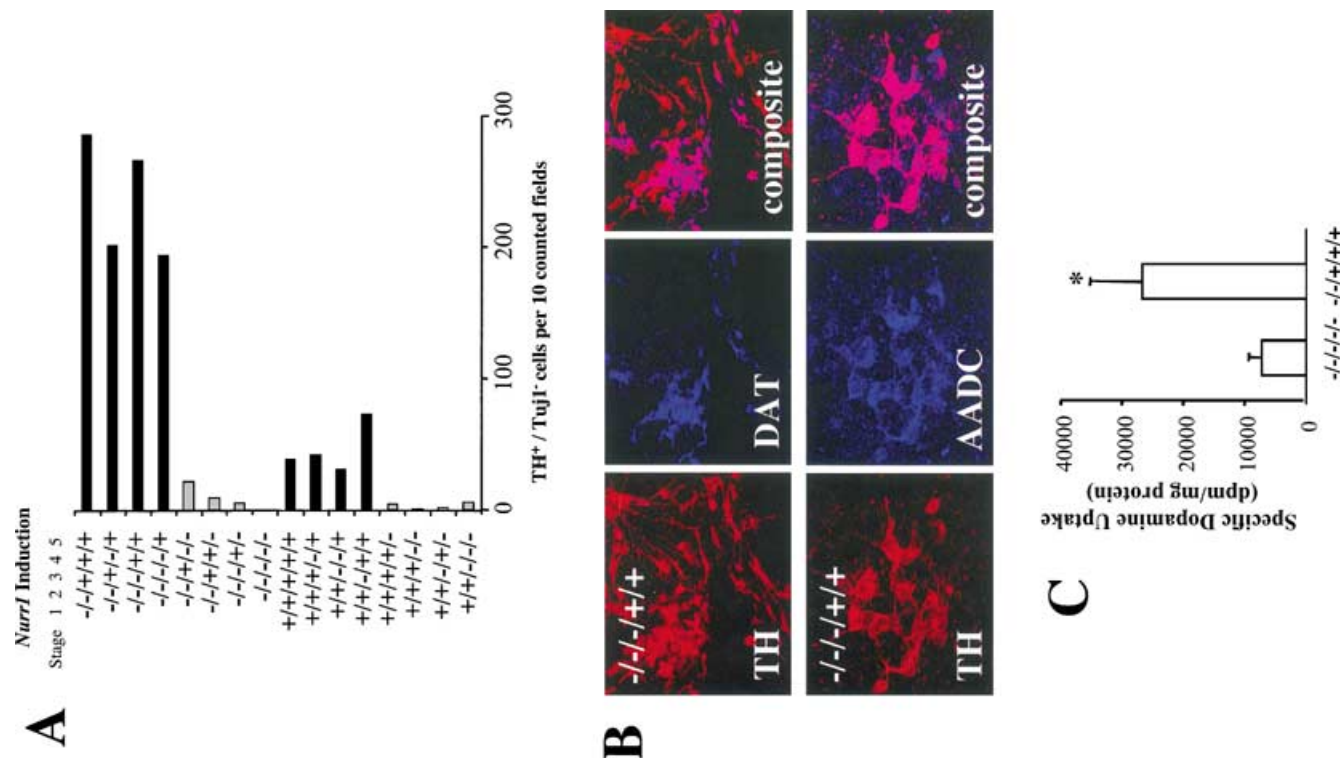


FIG. 7.

GATA4, HNF4 and Brachyury, however, remained present during differentiation when Nurr1 was induced early (stages 1 and 2), but not late (stages 3, 4, and 5).

Development of TH⁺ non-neuronal cellular phenotypes after Nurr1 induction

Using the various Nurr1-induction conditions (Fig. 4A), we evaluated whether exogenous Nurr1 influenced neurogenesis. Compared to naïve J1-rtTA ES cells, J1-rtTA-Nurr1 cells developed into similar numbers of Tuj1⁺ neurons in non-induced (-/-/-/-) conditions (Fig. 5B). In contrast, there was ~60% reduction of Tuj1⁺ neurons, when Nurr1 was induced starting at stage 3 (-/-/+ /+ /+) of the protocol (Fig. 5B), although the development of neural subpopulations, including Glu⁺, GABA⁺, ChAT⁺ neurons, or GFAP⁺ astrocytic cells, was not demonstrably altered (Fig. 5C).

To determine the effects of induced Nurr1 expression on the specification of a DA cellular phenotype, differentiated cells were examined for coexpression of TH and Tuj1. As in naïve J1-rtTA cells, TH⁺/Tuj1⁺ neurons developed from J1-rtTA-Nurr1 ES cell clones (Fig. 6A, upper row). However, when exogenous Nurr1 expression was induced starting from stage 3 (-/-/+ /+ /+), stage 4 (-/-/+ /+ /+) or stage 5 (-/-/+ /+ /+), new TH⁺/Tuj1⁻ cell populations appeared (Fig. 6A, lower row). These TH⁺ cells were negative for a variety of neural markers including the nuclear antigen NeuN for mature neurons (Fig. 6B), the embryonic neural cell adhesion molecule PSANCAM, GFAP for astrocytes, O4 or galactocerebrosidase C (GalC) for oligodendrocytes, as well as vesicle monoamine transporters (VMAT-1 and -2) and phenylethanolamine-N-methyltransferase (PNMT) for chromaffin cells (unpublished data). In addition, this TH⁺ non-neural cell population did not express the pan-ectodermal marker Cytokeratin or the mesodermal markers Myosin and Desmin (data not shown) and were also negative for the neural precursor marker Nestin (Fig. 6C) as well as the immature stem cell marker 'stage specific early antigen 1 (SSEA1)' (Fig. 6D). Notably, these TH⁺/Tuj1⁻ cells appeared already at the NP cell stage (unpublished data).

The TH⁺ non-neural cells had at least three different morphologies; large uni- or bipolar, clusters of round cells, and jagged-like cellular phenotypes (unpublished data). An analysis of these cells after *in vitro* differentiation demonstrated that the TH⁺ cell populations were present when exogenous Nurr1 was expressed late (stage 5, ND). Cell counts in the various Nurr1 induction conditions revealed high numbers of TH⁺ non-neural cell populations (31–286 cells per 10 counted fields), when Nurr1 was induced during stage 5 (Fig. 7A). However, only a few TH⁺/Tuj1⁻ cells (<10 cells per 10 counted fields) were detected in the absence of Nurr1 expression at stage 5, even when

Nurr1 had been induced through stages 1–4 (Fig. 7A). These data demonstrate that the TH⁺/Tuj1⁻ cells can be induced at precursor stages but required late (stage 5) Nurr1 expression for maintaining the TH phenotype.

The TH⁺ non-neural cell populations acquire a functional DA phenotype and are responsive to signalling molecules

ICC experiments revealed that the TH⁺/Tuj1⁻ cell populations expressed DA markers such as DAT and AADC (Fig. 7B) and confirmed the observed up-regulation of these markers as seen by RT-PCR (see Figs 4B and 5A). It is interesting to note that all of the TH⁺ cells also expressed AADC (Fig. 7B, lower row), whereas DAT was only coexpressed in a fraction of these cells (Fig. 7B, upper row). To gain insight about the functionality of the TH⁺ non-neuronal cells, we performed DA uptake assays. When compared to Nurr1 non-induced conditions, there was an ~3-fold increase of uptake in cell cultures when Nurr1 was induced starting at stage 3 (Fig. 7C). Cell counts of TH⁺ subpopulations developed from this culture condition showed high amounts of the TH⁺ non-neuronal phenotype (Fig. 8A) demonstrating that this cell population expressed functional DAT proteins.

We also tested the effects of the signalling molecules SHH and FGF8 (Lee *et al.*, 2000; Chung *et al.*, 2002) on the TH⁺ non-neuronal population (Fig. 8A). There was an approximately two fold increase in TH⁺/Tuj1⁻ cell numbers in the presence of SHH and FGF8 (Fig. 8A, right panel) indicating that the TH⁺/Tuj1⁻ cells were responsive to signalling factors.

Nurr1 induces glial cell line-derived neural growth factor (GDNF)-related gene expression

Expression of the signal transduction molecule Ret is diminished in midbrain DA neurons of Nurr1-deficient mice (Wallen *et al.*, 2001). We therefore analysed Ret expression in the J1-rtTA-Nurr1 cells under the different induction conditions. There was up-regulation of Ret gene expression in stage 3 Nurr1-induced cells and in differentiated cells (ND, stage 5) when Nurr1 was induced starting from stages 3, 4, and/or 5 (Fig. 8B). In addition, Ret was expressed in the TH⁺ non-neuronal cell population as shown by ICC (Fig. 8C). As Ret mediates signal transduction induced by GDNF (Airaksinen & Saarma, 2002), we also included the analyses of GDNF family receptors- α (GFR α) gene expression. There was a clear correlation between Ret and GFR α 1 expression when exogenous Nurr1 was expressed at stages 3, 4 or 5 of the protocol. Late (stage 4 and 5) induction of Nurr1 was also associated with up-regulation of the GFR α 2 splice isoforms a, b and c (Wong & Too, 1998) and the putative glycosylphosphatidylinositol (GPI) and transmembrane (TM) isoforms of GFR α 4 (Lindahl *et al.*, 2000).

Fig. 7. Characterization and functional analysis of the TH⁺/Tuj1⁻ cell populations. (A) Expression and maintenance of the TH⁺ non-neuronal cellular phenotypes require late induction of Nurr1. J1-rtTA-Nurr1 ES cells were differentiated using various induction conditions as indicated. After differentiation, TH⁺/Tuj1⁻ cells were counted. Data are shown for one representative ES cell clone and comprise total cell counts from 10 fields per coverslip for each individual differentiation experiment. (B) The TH⁺ non-neural cells coexpress TH (red) and DAT (blue) (upper row) or AADC (blue) (middle row). (C) DA uptake assay on differentiated J1-rtTA-Nurr1 cells developed from non-induced and stage 3 (-/-/+ /+ /+) Nurr1 induction conditions. DA uptake was measured as described in material and methods. There was a significant increase (**P* < 0.05) of DA uptake in the Nurr1 induced culture conditions. Shown is one out of three experiments performed on two individual cell clones.

Fig. 8. Effects of signalling molecules and Nurr1-induced expression of genes of trophic factors in cell differentiation. (A) Quantification of TH⁺/Tuj1⁺ and TH⁺/Tuj1⁻ cells developed from J1-rtTA-Nurr1 ES cells at different induction conditions and in the presence or absence of SHH and FGF8. Shown are representative data from one J1-rtTA-Nurr1 cell clone. #Significant differences in TH⁺/Tuj1⁻ cell counts comparing untreated with SHH/FGF8-treated culture conditions under stage 3 (-/-/+ /+ /+) and stage 4 (-/-/+ /+ /+) Nurr1 induction (#*P* < 0.05 as evaluated by ANOVA and Fisher's PLSD posthoc analysis). The increase of TH⁺/Tuj1⁻ cells in SHH/FGF8 minus conditions was not significant between stage 3 and stage 4 Nurr1 induction (**P* = 0.0626). (B) Expression of Ret and GFR α genes in J1-rtTA-Nurr1 cells at different stages (ES, NP, ND) of cell differentiation. ES cells were differentiated as schematically shown in Fig. 4A. Gene expression was analysed by RT-PCR and quantified as described in legend of Fig. 5A. (C) ICC of TH (red) and Ret (blue) expression in TH⁺/Tuj1⁻ cells developed from stage 3 (-/-/+ /+ /+) Nurr1 induction conditions.

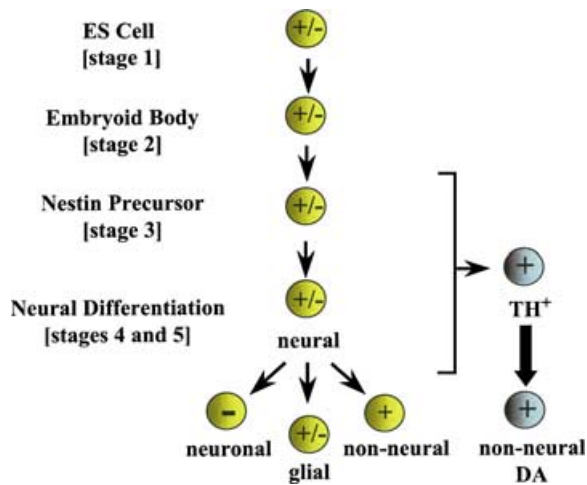


Fig. 9. Schematic presentation of expression patterns of an induced transgene in recombinant J1-rtTA ES cells, and the generation of TH⁺ non-neuronal cell populations in J1-rtTA-Nurr1 cell differentiation described in this study. One major characteristic of J1-rtTA ES cells is the differential expression of transgenes in ES cells, EBs, nestin precursors and astroglia and the lack of transgene expression in mature neurons (left side of panel and see Figs 2 and 3). In the right side of panel, the induction of a full non-neuronal DA phenotype (TH⁺) is induced when Nurr1 is expressed (+) at stages 3, 4, and/or 5 of ES cell differentiation.

These results demonstrate that Nurr1 can control not only the DA phenotype, but also GDNF-related gene expression.

Discussion

Here, we show that temporally induced Nurr1 in ES cell differentiation can regulate a DA cell type with functional DAT and its related trophic factors independent of a neuronal context (Fig. 9) providing evidence that an induced transcriptional activator can define a complete biochemical cellular and functional phenotype.

Using hrGFP-engineered ROSA26 J1-rtTA ES cells, we found that the GFP reporter gene was expressed in immature ES cells and neural precursors, in 5–10% of astrocytes, but not in mature neurons. This provided an opportunity to study the biological effects of induced Nurr1 expression independent of neurogenesis. Nurr1 is a transcriptional activator necessary for the specification of the mesencephalic DA phenotype in embryogenesis (Zetterstrom *et al.*, 1997; Castillo *et al.*, 1998; Saucedo-Cardenas *et al.*, 1998) and when constitutively over-expressed in ES cell clones, facilitates differentiation into mid-brain DA neurons (Chung *et al.*, 2002; Kim *et al.*, 2002). In the neuronal studies, Nurr1 requires a specific cellular and/or neuronal environment presumably providing ligands, cofactors and/or certain transcriptional conditions (Sakurada *et al.*, 1999; Wagner *et al.*, 1999; Chung *et al.*, 2002; Kim *et al.*, 2003b). Here, using the J1-rtTA-Nurr1 ES cell line, we found that cellular Nurr1 expression at late developmental stages (but not at ES and EB stages) induced the expression of most known DA markers also in non-neuronal cell populations. Removing Nurr1 induction during the last differentiation stages diminished the DA phenotypic markers, indicating that maintenance of the DA phenotype required continuous Nurr1 expression. In these non-neuronal cells, Nurr1 induction was sufficient to produce a combination of typical characteristics of a functional molecular and biochemical DA phenotype, such as DA synthesizing enzymes (TH, AADC), functional DAT, and known DA-associated markers (calbindin). This is consistent with studies in Nurr1-deficient mice confirming that Nurr1 is essential for expressing the full dopaminergic phenotype

(Saucedo-Cardenas *et al.*, 1998; Wallen *et al.*, 1999; Witta *et al.*, 2000) and with a suggested instructive role in controlling DA synthesis and storage in MN9D cells (Hermanson *et al.*, 2003). As expected, Nurr1 did not have a significant effect on the expression of other transcriptional activators, such as Pitx3 and Imx1b, which are involved in the development of midbrain mesencephalic neurons (Pitx3) and in a separate pathway for DA specification (Imx1b) (Smidt *et al.*, 2000). This result is consistent with the view that Pitx3 and Imx1b act independently from Nurr1 in defining the midbrain mesencephalic and the DA phenotype (Smidt *et al.*, 2000). Our studies also show that Nurr1 did not induce homeobox genes such as Otx1, Otx2 and GBX2 that are important in organizing the cephalic neural primordium, or those involved in controlling the differentiation of the mid- and hindbrain system, such as En-1 (Rhinn & Brand, 2001). Taken together, we demonstrate in the J1-rtTA ES cell system that Nurr1 is a late determining transcriptional activator for the DA phenotype, while not involved in ventralization and genesis of neuroepithelial cells in the neural plate.

Furthermore, our results show that Nurr1 induces the expression of the tyrosine kinase receptor Ret, which mediates signal transduction by GDNF and related neurotrophic factors (Airaksinen & Saarma, 2002). In Nurr1-deficient mice, Ret expression is reduced (Zetterstrom *et al.*, 1997; Wallen *et al.*, 2001). Our studies demonstrate that Nurr1 not only induced Ret but also correlated with GFR α gene expression. Thus, Nurr1-induced receptors may be essential for the survival of fetal mesencephalic precursors in the developing ventral-midbrain in mice (Saucedo-Cardenas *et al.*, 1998). It is interesting to note, that there was a close relationship between Nurr1, Ret and GFR α 1 gene expression at earlier stages of cell differentiation, whereas an increase of transcriptional splice isoforms of GFR α 2 (Wong & Too, 1998) and GFR α 4 (Lindahl *et al.*, 2000) were detected only at later stages of Nurr1 induction. In summary, Nurr1 can induce receptors relevant for the trophic action of GDNF- (GFR α 1), Neurturin- (GFR α 2) and Artemin- (GFR α 4), further supporting its possible role in mediating cell survival.

In several studies, it has been reported that Nurr1 can be induced in non-neuronal cells, e.g. in primary mouse osteoblasts (Tetradis *et al.*, 2001), in synovial tissue (Murphy *et al.*, 2001; McEvoy *et al.*, 2002) and in corticotroph cells (Kovalovsky *et al.*, 2002). Although a function of Nurr1 was not determined, Nurr1 was suggested to be involved in several signalling pathways, such as mediating a potential cross-talk mechanism between PTH and vitamin D, or other steroid hormone signalling in regulating bone metabolism (Tetradis *et al.*, 2001), CREB-1 and NF- κ B in inflammatory processes (McEvoy *et al.*, 2002) and/or calcium/calmodulin kinase II and MAPK in proopiomelanocortin expression (Kovalovsky *et al.*, 2002). In our study, induction of Nurr1 in J1-rtTA-Nurr1 ES cell differentiation produced a DA phenotype. These cells appeared at the neural precursor stage, were mitotic and responded to signalling molecules, and lacked the expression of the embryonic marker SSEA1 and the precursor marker Nestin. The DA cells were negative for Desmin and Cytokeratin, two markers for mesodermal or ecto- and endodermal derived cellular phenotypes, respectively. When Nurr1 was expressed at ES cell stage and/or during EB formation, this led to maintained expression of the early endodermal transcription factor GATA4 (Soudais *et al.*, 1995), the late endodermal factor hHNF4 (Li *et al.*, 2000) and Brachyury, a direct factor in the early events of mesoderm formation (Wilkinson *et al.*, 1990). Notably, transcripts of CK-17, an ectodermal marker of undifferentiated mouse ectoderm (Mcgowan & Coulombe, 1998), was not present after *in vitro* differentiation. Therefore, early expression of Nurr1 could have favoured meso- and endodermal development by up-regulating Ret expression, thus enhancing trophic support in these

early cell populations. In contrast, stage 3, 4, or 5 induction of Nurr1 may have enhanced Ret-mediated proliferation and survival of a later intermediate non-neuronal precursor, which then attained the DA phenotype. These conditions could have suppressed or inhibited the development of other cellular subtypes from all three germ-layers, including some neurons.

The J1-rtTA ES cells lacked transgene expression in mature neurons. A possible explanation for this could be that genetic modification of ES cells at the ROSA26 locus influenced their capacities of gene expression in neurons. Although, ROSA26-lacZ mice ubiquitously expressed the trapped transgene including in the brain, expression was not detected in olfactory bulb granule cells indicating that some neural cells may not express from the ROSA26 locus (Zambrowicz *et al.*, 1997). In our *in vitro* differentiation experiments, the J1-rtTA ES cells showed a reduction in neurogenesis and neurons were slightly modified in their morphologies when compared to the WT J1 cells.

Other explanations include gene-regulating mechanisms in ES cell differentiation, such as DNA methylation (Lei *et al.*, 1996). However, treatment with the demethylation agent 5'-aza-2'-deoxycytidine did not increase induced GFP expression (data not shown) indicating that methylation was not involved in silencing of the rtTA and/or GFP reporter gene expression.

Other possible mechanisms include the function of the cellular phenotype, which might not have provided the cellular machinery to activate the Tet-System. During differentiation, a fraction of the Nestin⁺ precursors, immature neurons and ~5–10% of astrocytes were able to express the GFP reporter gene indicating that cells from the neural lineage were not per se deprived of inducibly expressing genes from the Tet-Response Element promoter. This is consistent with results from other studies demonstrating the functionality of the Tet-System in neurons (Harding *et al.*, 1997; Jaworski *et al.*, 2000; Kafri *et al.*, 2000; Ralph *et al.*, 2000). In our study, we can not rule out the possibility that rtTA expression was inhibited in mature neural phenotypes. However, it seems that yet undetermined factors might have been involved in blocking transgene regulation in the J1-rtTA ES cell lines.

In conclusion, we have shown that ROSA26-engineered J1-rtTA ES cells do not express transgenes in neurons and therefore provide a tool for expressing (neuronal) factors independent of their cell type specification. We show that Nurr1 is not necessarily linked to neuronal differentiation providing evidence and insight for possible functions of transcription factors in controlling the biochemical identity of a cellular phenotype independent of a role in cell fate specification. Notably, recent data by Wang *et al.* (Wang *et al.*, 2003) have highlighted that Nurr1 belongs to a unique structural class of nuclear receptors with ligand-independent function. Our data illuminate and add to the interpretations by Wagner *et al.* (Wagner *et al.*, 1999), concluding that cellular context-dependent factors were required for the full 'DA' biochemical phenotype to be established by Nurr1 in neurons derived from some cell lines. Our study also demonstrates that removal of induced Nurr1 in DA phenotypic cells, even in the non-neuronal context, results in the down-regulation of DA markers reflecting its normal role in vivo (Castillo *et al.*, 1998; Saucedo-Cardenas *et al.*, 1998; Witta *et al.*, 2000; Wallen *et al.*, 2001) and the adult brain (Backman *et al.*, 1999; Chen *et al.*, 2001; Xu *et al.*, 2002; Backman *et al.*, 2003; Le *et al.*, 2003). The observation that Nurr1 induces a DA biochemical phenotype independent of a neuronal phenotype at late stages of cell development is consistent with a new and revised view of Nurr1 function in DA biochemical and trophic specification in the midbrain. This view of Nurr1 may also explain neurotrophic-dependent changes seen in both the developing and adult nervous system, including DA cells affected in Parkinson's disease and schizophrenia.

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Abbreviations

AADC, aromatic L- amino acid decarboxylase; AHD2, aldehyde-dehydrogenase; ChAT, choline acetyl transferase; CK-17, Cytokeratin 17; DA, dopaminergic; DAT, dopamine transporter; d.b.h., decarboxyhydroxylase; EB, embryoid body; ES, embryonic stem; FGF, fibroblast growth factor; GABA, gamma aminobutyric acid; GalC, galactocerebrosidase C; GDNF, glial-derived nerve growth factor; GFAP, glial fibrillary acidic protein; GFR, GDNF-family receptor; GFP, green fluorescent protein; Glu, glutamate; GluT, glutamate transporter; GPI, glycosylphosphatidylinositol; HNF4, hepatic nuclear factor 4; ICC, immunocytochemistry; ND, neural differentiation; NeuN, neuronal nuclei; NP, neural precursor; PNMT, phenylethanolamine-N-methyltransferase; PSAN-CAM, antiembryonic neural cell adhesion molecule; rtTA, reverse transactivator; SSEA1, stage specific early antigen 1; SSH, sonic hedgehog; TH, tyrosine hydroxylase; TM, transmembrane; TPH, tryptophan hydroxylase; TRE, tet-response element; VMAT, vesicle monoamine transporter.

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