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Original Article

The constitutive and inducible expression of Nurr1, a key regulator of dopaminergic neuronal differentiation, in human neural and non-neural cell lines

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Nur-related factor 1 (Nurr1), nerve growth factor-induced gene B (NGFI-B) and neuron-derived orphan receptor-1 (NOR-1) constitute the orphan nuclear receptor subfamily of transcription factors. Previous studies showed that midbrain dopaminergic neuronal precursor cells failed to differentiate in Nurr1-deficient mice. To investigate a role of Nurr1 in human neuronal function, Nurr1 mRNA expression was studied in human neural cell lines by RT-PCR and northern blot analysis. Nurr1, NGFI-B and NOR-1 mRNA were coexpressed in all human neural and non-neural cell lines under the serum-containing culture condition, except for SK-N-SH neuroblastoma, in which Nurr1 mRNA was undetectable. The levels of Nurr1, NGFI-B and NOR-1 mRNA were elevated markedly in NTera2 teratocarcinoma-derived neurons (NTera2-N), a model of differentiated human neurons, following a 1.5 or 3 h-exposure to 1 mM dibutyryl cyclic AMP or 100 nM phorbol 12-myristate 13-acetate. NGFI-B mRNA levels were also elevated in NTera2-N cells by exposure to 100 ng/mL brain-derived neurotrophic factor (BDNF). To identify Nurr1-target genes, the mRNA expression of 27 genes potentially involved in dopaminergic neuronal differentiation and survival, including BDNF, glia-derived neurotrophic factor, their receptors, tyrosine hydroxylase and α-synuclein, were studied in HEK293 cells following overexpression of Nurr1. None of these genes examined, however, showed significant changes. These results indicate that Nurr1, NGFI-B and NOR-1 mRNA are expressed constitutively in various human neural and non-neural cell lines under the serum-containing culture condition, and their levels are up-regulated in human neurons by activation of protein kinase A or protein kinase C pathway, although putative coactivators expressed in dopaminergic neuronal precursor cells might be required for efficient transcriptional activation of Nurr1-target genes.

Key words: dopaminergic neurons, NGFI-B, NOR-1, NTera2, Nurr1.

INTRODUCTION

Nur-related factor 1 (Nurr1, also termed NR4A2), nerve growth factor-induced gene B (NGFI-B; NR4A1), and neuron-related orphan receptor-1 (NOR-1; NR4A3) are three closely related, highly homologous nuclear transcription factors of the steroid/thyroid hormone receptor superfamily, and they are designated as orphan nuclear receptors (ONR) because of lack of their cognate ligands.[1,2] They are encoded by immediate early genes which are rapidly induced by exposure of cells to the serum, growth factors, cytokines, and peptide hormones, suggesting a biological role of ONR in intracellular signal transduction involved in cell growth, differentiation and survival.[3–6]

Increasing evidence indicates that Nurr1 plays a pivotal role in terminal differentiation of ventral mesencephalic dopaminergic precursor neurons into a complete dopaminergic phenotype at later stages of development.[7–17] Nurr1 is expressed most abundantly in neurons of limbic areas and the ventral midbrain in the mouse and rat central nervous system (CNS).[7–11] Nurr1, not NGFI-B or NOR-1, is expressed in dopaminergic neurons in the substantia nigra

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(SN, A9) and the ventral tegmental area (VTA, A10) in the developing and adult rodent CNS. Nurr1 expression is identified in the mouse midbrain, starting from embryonic day 10.5, that is, 1.5 days earlier than the appearance of dopaminergic neurons. Most importantly, Nurr1 knockout ($Nurr1^{-/-}$) mice die shortly after birth because of hypoactivity, due to selective agenesis of dopaminergic neurons in both SN and VTA.[12–17] Although a population of dopaminergic precursor cells expressing the early dopaminergic neuronal markers such as aldehyde dehydrogenase 2 (AHD2), engrailed (En), and a homeodomain transcription factor Ptx-3 develops in the ventral midbrain of Nurr1-deficient mice, they could not terminally differentiate into fully mature dopaminergic neurons characterized by the expression of tyrosine hydroxylase (TH), L-aromatic amino acid decarboxylase (AADC), dopamine transporter (DAT), vesicular monoamine transporter, and dopamine receptors (DR) D1 and D2. Overexpression of Nurr1, in combination with a diffusable factor derived from type 1 astrocytes isolated from ventral mesencephalon, induces neural stem cells to develop into midbrain dopaminergic neurons.[18] Heterozygous $Nurr1^{+/-}$ mice survive postnatally without obvious movement abnormalities, but they exhibit enhanced vulnerability to the selective dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP).[19] All of these observations suggest that Nurr1 acts as a key transcriptional factor crucial for a set of genes which are activated during differentiation of midbrain dopaminergic neurons and maintenance of their function in adulthood. However, the relevance of these findings to the pathogenesis of human neurodegenerative diseases such as Parkinson's disease remains to be established because most previous studies have focused on induction of Nurr1 expression in rodent cells.[20]

The ONR family has a structure composed of an N-terminal variable domain containing the activation function 1 (AF1) region, a central DNA-binding domain (DBD) with two conserved zinc finger motifs and sequences termed P-box, D-box, T-box and A-box, and a C-terminal ligand-binding domain (LBD) containing a leucine-zipper motif and the activation function 2 (AF2) region.[21,22] Nurr1 binds to a *cis*-acting regulatory sequence termed the NGFI-B-responsive element (NBRE; 5′AAAGGTCA3′) as a monomer, and also interacts with a direct repeat of the consensus half-site sequence (5′AGGTCANNNAAAGGTCA3′; DR5) as a heterodimer with the retinoid X receptor (RXR), a universal heterodimeric partner for the retinoic acid (RA) receptor, thyroid hormone receptor, vitamin D receptor, and peroxisome proliferator-activated receptor.[9,23,24] NGFI-B can also heterodimerize with RXR, whereas NOR-1 is unable to form a stable heterodimer with RXR. Furthermore, Nurr1 has a capacity to interact with the Nur77 response element (NurRE), a palindromic element containing a six-nucleotide spacing between two consensus half-site sequences as a Nurr1 homodimer or a heterodimer with NGFI-B or NOR-1.[25,26] At present, only a few Nurr1 target genes have been identified, one of which is the TH gene that contains two NBRE-like sequences in its promoter region.[27,28]

The aim of this study is (1) to investigate Nurr1 mRNA expression in human neural cell lines (2) to clarify upstream signals regulating Nurr1 mRNA expression in human neurons, and (3) to identify the target genes regulated by Nurr1 in human cell lines.

METHODS

Human neural and non-neural cell lines and tissues

The human neural and non-neural cell lines were obtained from the RIKEN Cell Bank (Tsukuba, Japan) and the American Type Culture Collection (Rockville, MD, USA).[29–32] They were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL of penicillin and 100 µg/mL of streptomycin (feeding medium). NTera2/c.D1 (NTera2) is a pluripotent human teratocarcinoma-derived clonal cell line. When maintained under standard culture conditions, NTera2 cells have characteristics of undifferentiated neuronal progenitor cells (NTera2-U), while they differentiate into postmitotic neurons (NTera2-N) after a 4-week treatment with 10^{-5} M *all trans* retinoic acid (RA) (Sigma, St. Louis, MO, USA) followed by the procedure of repeated replating, as described previously.[29–32] The source of human cerebral and cerebellar tissues, peripheral nerve, and skeletal muscle was specified in a previous study.[30]

For treatment of NTera2-N cells with growth/differentiation factors, the cells were washed once with the serum-free DMEM and incubated for 48 h in the serum-free DMEM/F-12 medium supplemented with insulin, transferrin, and selenium (ITS-G; Gibco-BRL/Invitrogen, Carlsbad, CA, USA) (experiment medium), to exclude the effects of the serum. Then, they were incubated further for 1.5 or 3 h in the serum-free experiment medium, supplemented with 100 ng/mL of recombinant human β-nerve growth factor (NGF; PeproTech, London, UK), brain-derived neurotrophic factor (BDNF; PeproTech), or glia-derived neurotrophic factor (GDNF; PeproTech), or 100 nM of phorbol 12-myristate 13-acetate (PMA; Sigma), an activator of protein kinase C (PKC), 1 mM of dibutyryl cyclic AMP (dbcAMP; Sigma), an activator of protein kinase A (PKA), either in the presence or absence of

10 μg/mL cycloheximide (CHX; ICN Pharmaceuticals, Costa Mesa, CA, USA), or 200 μM of SKF-82958 hydrobromide (Sigma), a full D1DR agonist, followed by processing for RNA preparation.

RT-PCR and Southern blot analysis

Total RNA was extracted from the cells and tissues by the acid guanidinium thiocyanate-phenol-chloroform method using TRIZOL reagent (Invitrogen). DNase-treated RNA samples were processed for cDNA synthesis using oligo$(dT)_{12–18}$ primers and SuperScript II reverse transcriptase (Invitrogen). cDNA was amplified by polymerase chain reaction (PCR) using a panel of sense and antisense primer sets listed in Table 1. PCR was carried out in 25 μL of reaction mixture containing Taq DNA polymerase buffer and 0.625 U HotStar Taq DNA polymerase (Qiagen, Valencia, CA, USA). The amplification program consisted of an initial denaturating step at 95 °C for 15 min, followed by a denaturing step at 94 °C for 1 min, an annealing step at 60 °C for 40 s and an extension step at 72.9 °C for 50 s for 40 cycles, except for the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene that was amplified for 30 cycles. For Southern blot analysis, the PCR products separated on a 1.5% agarose gel were transferred onto a nylon membrane. The membranes were hybridized at 60 °C overnight with the internal oligonucleotide probe specific for the Nurr1 PCR product (5′TCGAAGCGCATCTGGCAACTAGAC3′) labeled with digoxigenin (DIG)-11-dUTP by the DIG oligonucleotide tailing kit (Roche Diagnostics, Mannheim, Germany) according to the methods described previously.[29,30,32] The specific reaction was visualized by the DIG chemiluminescence detection kit (Roche Diagnositics).

Northern blot analysis

For northern blot analysis, 3 μg of total RNA separated on a 1.5% agarose-6% formaldehyde gel was transferred onto a nylon membrane. After prehybridization, the membranes were hybridized at 54 °C overnight with the DIG-labeled DNA probe synthesized by the PCR DIG probe synthesis kit (Roche Diagnositics) using the specific primer sets (Table 1), followed by re-hybridization with the DIG-labeled human β-actin antisense RNA probe (Roche Diagnositics) as described previously.[31–33] The specific reaction was visualized on Kodak X-OMAT AR X-ray films by the DIG chemiluminescence detection kit (Roche Diagnositics). The densitometric analysis was performed on an imaging system using an NIH image Version 1.61 software. The levels of Nurr1, NGFI-B, and NOR-1 mRNA were standardized against the corresponding β-actin mRNA signals detected on the identical blots as described previously.[31–33]

Transfection and expression of Nurr1 gene

The entire open reading flame (ORF) of Nurr1 gene (1797 bp) was amplified by PCR using PfuTurbo DNA polymerase (Stratagene, La Jolla, CA, USA) and the sense and antisense primer set (5′ATGCCTTGTGTTCAGGCGCAGTAT3′ and 5′TTAGAAAGGTAAAGTGTCCAGGAA3′). Then, it was cloned in a mammalian expression vector pcDNA4/HisMax-TOPO (Invitrogen), which contains an N-terminal Xpress tag for detection of the recombinant protein. The orientation of the cloned genes was verified by direct sequencing analysis. Either the vector containing Nurr1 ORF or the vector pcDNA4/HisMax-TOPO/LacZ (Invitrogen) was transfected by using Lipofectamine Plus reagent (Invitrogen) in a battery of human neural and non-neural cell lines, which were incubated in the serum-free experiment medium for 24 h before transfection. After incubating the cells in the serum-free experiment medium for a further 96 h, they were processed for preparation of both total RNA and protein. The efficiency of transfection and expression was determined in the cells transfected with pcDNA4/HisMax-TOPO/LacZ by β-Gal staining kit (Invitrogen).

Western blot analysis

To prepare total protein extract, the cells were homogenized in RIPA lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS, and a cocktail of protease inhibitors; Roche Diagnositics), followed by centrifugation at 15 000 rpm. for 20 min at room temperature (RT). The supernatants were collected and 100 μg of protein extract was separated on an 8% SDS-PAGE gel as described previously.[32,34] They were then transferred onto a nitrocellulose membrane followed by immunolabeling for 1 h at RT with a mouse monoclonal antibody against the Xpress epitope (1 : 4000; Invitrogen). Then, the membranes were incubated for 1 h at RT with HRP-conjugated antimouse IgG (1 : 1000; Santa Cruz Biotechnology, Santa Cruz, CA). The specific reaction was visualized by the enhanced chemiluminescence (ECL) western blot detection system (Amersham Biosciences, Piscataway, NJ, USA). After the antibodies were stripped by incubation of the membranes in stripping buffer (62.5 mM Tris-HCl, pH 6.7, 2% SDS, 100 mM 2-mercaptoethanol) at 50 °C for 30 min, they were processed for relabeling with a rabbit polyclonal antibody against the mouse Nurr1 protein (1:1000; M-196, Santa Cruz Biotechnology) or with a goat polyclonal antibody against the human 60-kDa heat shock protein (HSP60), a housekeeping gene product (1:2000; N-20, Santa Cruz Biotechnology), followed by incubation with HRP-conjugated antirabbit or antigoat IgG (1:2000, Santa Cruz Biotechnology).

Table 1 Primer Sequences Used for PCR

Genes	GenBank Accession No.	Sense	Antisense	Product size (bp)
Nurr1	X75918	5′CGGACAGCAGTCCTCCATTAAGGT3′	5′CTGAAATCGGCAGTACTGACAGCG3′	712
NGFI-B	D49728	5′GTCCTCCAGTGGCTCTGACTACTA3′	5′CTTCAGGCTGTCTGTTCGGACAAC3′	658
NOR-1	D78579	5′TCTTGTCCGAGCTTTAACAGACTC3′	5′ACAGGCAGGCTAAGGCTTGGATAT3′	410
RXRα	X52773	5′ATCTTTGACAGGGTGCTGACGGAG3′	5′TCTCTTAGGCAGAGCAGTGACAGG3′	505
RORα	L14611	5′ATTCCATGCAAGATCTGTGGAGAC3′	5′CTGATGCTGGTGTGTAGTCACATA3′	544
NGF	V01511	5′CCAAGGGAGCAGCTTTCTATCCTGG3′	5′TTGTGTGGAGGGTCTGACTGACGCT3′	342
BDNF	M61176	5′CAAACATCCGAGGACAAGGTGGCTT3′	5′CTTTTGTCTATGCCCCTGCAGCCTT3′	535
NT3	M37763	5′TTCTCGCTTATCTCCGTGGCATCC3′	5′GGCAGGGTGCTCTGGTAATTTTCCT3′	167
GDNF	NM_000514	5′ATGTCACTGACTTGGGTCTGGGCTA3′	5′CAGATACATCCACACCTTTTAGCGG3′	259
LNGFR	M14764	5′AGCCAACCAGACCGTGTGTG3′	5′TTGCAGCTGTTCCACCTCTT3′	663
trkA	M23102	5′CCATCGTGAAGAGTGGTCTC3′	5′GGTGACATTGGCCAGGGTCA3′	476
trkB	S76473	5′AGGGCAACCCGCCCACGGAA3′	5′TTGGTGGCCTCCAGCGGCAG3′	638
trkC	S76475	5′ACGCCAGGCCAAGGGTGAGCT3′	5′TTCATGACCACCAGCCACCAC3′	567, 609
GDNFRα	U59486	5′GACTGTGTGAAAGCCAGCGATCAG3′	5′TTCCAGGAGGTCATTTCCCTGCAG3′	270
GDNFRβ	U93703	5′ATCTTGGCAAACGCCTTCTGCCTC3′	5′AGATGTAGGAGGAGCGCAGCTTCT3′	544
RET	M57464	5′GGGATTAAAGCTGGCTATGGCACC3′	5′CAGGATCTTGAAGGCATCCACGGA3′	336
FGFR-1	M34641	5′TCACAGCAACACTCTGCACCGCTA3′	5′ACGACATCCAGCTGGTATGTGTGG3′	697
D1DR	X58987	5′ATCATGAGCCACGAGGCTCCATCT3′	5′TGTTGGAAAGCAGCAGAGGGCTCT3′	352
D2DR	M29066	5′GCAAGCGAGTCAACACCAAACGCA3′	5′CCATTGGGCATGGTCTGGATCTCA3′	388
c-fos	K00650	5′CGATGATGTTCTCGGGCTTCAACG3′	5′CGCTTGGAGTGTATCAGTCAGCTC3′	503
PAG	X67951	5′CCACAGCTGTTATGCCAGATGGTC3′	5′CAGGCTTGATGGTATCACTGCCAG3′	510
α-synuclein	L08850	5′GCCATTCGACGACAGTGTGGTGTA3′	5′TGAGTGGGGGCAGGTACAGATACT3′	628
synphilin-1	AF076929	5′AGACGATCCCAGAACTGTGCCGAA3′	5′TGCTGGTCGTGGATGACAGCTGAT3′	610
synaptophysin	X06389	5′TCCGCCATCTTCGCCTTTGCCACA3′	5′CCCAGCCTGTCTCCTTAAACACGA3′	580
GAP-43	M25667	5′GACGAGACAACCATGCTGTGCTGT3′	5′GTTCTCTTCAGCTTGGCTGCTCTC3′	636
DAD1	D15057	5′ACCGGAGTACCTTGCGTGCAGTTA3′	5′CTGACACACAGTGAACTCTGGGCT3′	547
CRABP-II	M68867	5′TGCCCAACTTCTCTGGCAACTGGA3′	5′ATCTGGGCTCTTGCAGCCATTCCT3′	635
Pax-5	M96944	5′ATTATCCGACTCCTCGGACCAGCA3′	5′CGGAGACTCCTGAATACCTTCGTC3′	605
TH	Y00414	5′GTCCCGAGCTGTGAAGGTGTTTGA3′	5′ATTGTCTTCCCGGTAGCCGCTGAA3′	517
HSP27	X54079	5′TTCTGAGCAGACGTCCAGAGCAGA3′	5′GATGGTGATCTCGTTGGACTGCGT3′	576
HSP70	M11717	5′GACCAAGATGAAGGAGATCGCCGA3′	5′GAAGAAGTCCTGCAGCAGCTTCTG3′	694
G3PDH	J04038	5′CCATGTTCGTCATGGGTGTGAACCA3′	5′GCCAGTAGAGGCAGGGATGATGTTC3′	251

Nurr1, nur-related factor 1; NGFI-B, nerve growth factor-induced gene B; NOR-1, neuron-derived orphan receptor-1; RXRα, retinoid× receptor alpha; RORα, retinoic acid (RA) receptor-related orphan receptor alpha; NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; NT3, neurotrophin-3; GDNF, glia-derived neurotrophic factor; LNGFR, low-affinity NGF receptor; trk, tyrosine kinase receptor; GDNFR, GDNF receptor; RET, receptor tyrosine kinase; FGFR-1, fibroblast growth factor receptor-1; D1DR, D1 dopamine receptor; D2LDR, long form of D2 dopamine receptor; PAG, proliferation-associated gene; GAP-43, growth associated protein-43; DAD1, defender against apoptotic cell death-1; CRABP-II, cellular RA-binding protein-II; Pax-5, paired box gene-5; TH, tyrosine hydroxylase; HSP, heat shock protein; G3PDH, glyceraldehyde-3-phosphate dehydrogenase.

RESULTS

Nurr1, NGFI-B and NOR-1 mRNA are expressed constitutively in a variety of human neural and non-neural cell lines under the serum-containing culture condition

First, Nurr1, NGFI-B and NOR-1 mRNA expression was studied in human neural cell lines by RT-PCR analysis. Nurr1 mRNA was identified in almost all human neural and non-neural cell lines examined, when incubated in the feeding medium containing 10% FBS. They include IMR-32 neuroblastoma, U-373MG astrocytoma, NTera2-U cells, NTera2-N cells, HeLa cervical carcinoma, HEK293 embryonal kidney cells, and K-562 erythroleukemia (Fig. 1, panel (a), lanes 2,4–9), except for nonexpression of Nurr1 mRNA in SK-N-SH neuroblastoma (Fig. 1, panel (a), lane 3). Nurr1 mRNA was also identified in cultured skin fibroblasts (SF), peripheral blood mononuclear cells (PBMC), and adult human tissues of the cerebrum, cerebellum, peripheral nerve and skeletal muscle (Fig. 1, panel (a), lanes 10–15). The specificity of PCR was verified by Southern blot analysis using the Nurr1 PCR product-specific internal oligonucleotide probe (Fig. 1, panel (b), lanes 2–15). Both NGFI-B and NOR-1 transcripts were also expressed at variable levels in all human neural and non-neural cells and tissues examined (Fig. 1, panels (c) and (d), lanes 2–15). The levels of

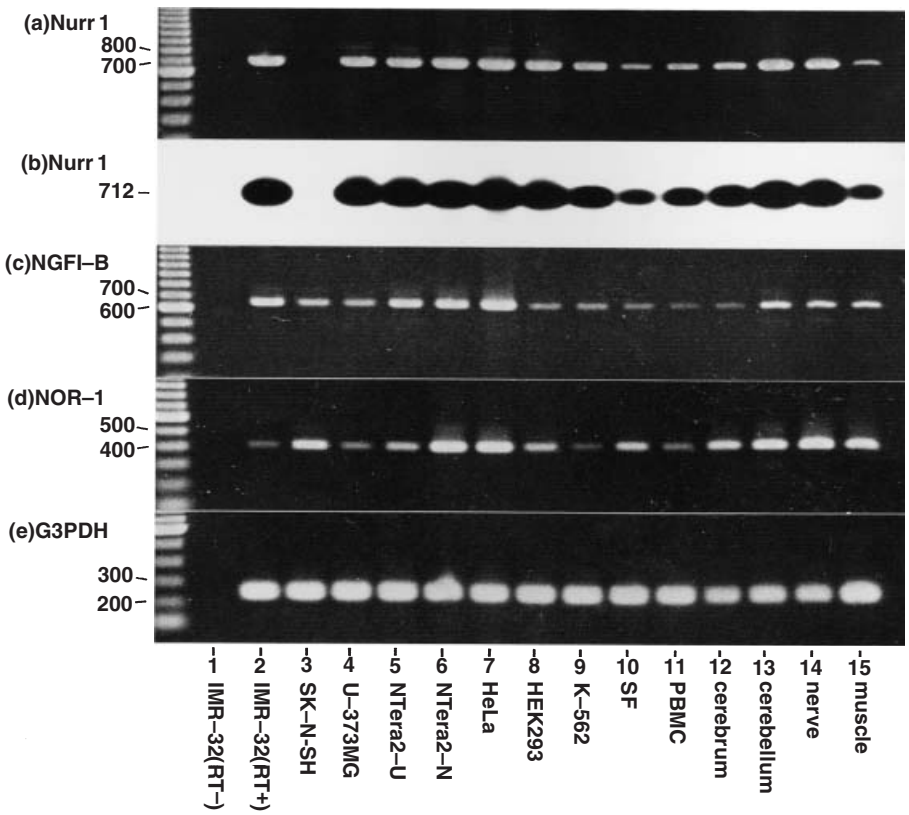

Fig. 1 Nurr1, NGFI-B and NOR-1 mRNA expression in human neural and non-neural cell lines. Human neural and non-neural cell lines were incubated in the feeding medium containing 10% FBS. 50 ng of cDNA was amplified for 40 cycles by PCR using the primer sets (Table 1) specific for the genes encoding Nur-related factor 1 (Nurr1): (**a,b**), nerve growth factor-induced gene B (NGFI-B); (**c**), or neuron-related orphan receptor-1 (NOR-1); (**d**), except for the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene; (**e**) amplified for 30 cycles. The amplified products separated on a 1.5% agarose gel were transferred onto a nylon membrane and hybridized with the Nurr1 PCR product-specific internal oligonucleotide probe. Panels (**a,c,d,e**) are ethidium bromide staining of the gels, while panel (**b**) is Southern blot of the gel corresponding to panel (**a**). Lanes (1–15) represent the following human cells and tissues:

1) IMR-32 neuroblastoma processed for PCR omitting the reverse transcription (RT) step
2) IMR-32 neuroblastoma processed for PCR including the RT step
3) SK-N-SH neuroblastoma
4) U-373MG astrocytoma
5) NTera2 teratocarcinoma maintained in the undifferentiated state (NTera2-U)
6) NTera2-derived differentiated neurons (NTera2-N)
7) HeLa cervical carcinoma
8) HEK293 embryonal kidney cells
9) K-562 erythroleukemia cells
10) cultured skin fibroblasts (SF)
11) peripheral blood mononuclear cells (PBMC)
12) cerebrum
13) cerebellum
14) peripheral nerve of the cauda equina, and
15) skeletal muscle. The DNA size marker (bp) is shown on the left.

G3PDH mRNA, a housekeeping gene, were almost constant among the cells and tissues examined (Fig. 1, panel (e), lanes 2–15), while no products were amplified when total RNA was processed for PCR without inclusion of the reverse transcription (RT) step, confirming that a contamination of genomic DNA was excluded (Fig. 1, panels (a)-(e), lane 1). These results indicate that Nurr1, NGFI-B and NOR-1 mRNA are coexpressed constitutively in various human neural and non-neural cell lines under the serum-containing culture condition, suggesting their fundamental role in diverse cell types.

Neurotrophic factor receptors are expressed in NTera2-N cells, a model of differentiated human neurons

NTera2-N cells exhibiting extensive neurite outgrowth represent a model system of differentiated human neurons in culture.[29–32] The transcripts coding for a panel of neurotrophic factor receptors were identified in NTera2-N cells by RT-PCR analysis. They include the low-affinity NGF receptor (LNGFR), tyrosine kinase receptors (trkA, trkB, and trkC), GDNF receptor-α (GDNFRα), and the receptor tyrosine kinase (RET) (Fig. 2, lanes 2,4,6,8,10,12), suggesting that NTera2-N cells are capable of responding to NGF, BDNF, NT3 and GDNF. Furthermore, NTera-N cells expressed mRNA encoding a battery of dopaminergic neuron-associated molecules, such as synaptophysin, α-synuclein, synphilin-1, D1 dopamine receptor (D1DR), and the long form of D2 dopamine receptor (D2LDR) (Fig. 2, lanes 14,16,18,20,22). By contrast, no products were amplified when total RNA was processed for PCR omitting the RT step (Fig. 2, lanes 1,3,5,7,9,11,13,15,17,19,21).

Nurr1, NGFI-B and NOR-1 mRNA expression is up-regulated in NTera2-N cells by exposure to PKA and PKC activators

In the next step, the extracellular stimuli inducing Nurr1, NGFI-B, and NOR-1 mRNA expression were studied by northern blot analysis in NTera2-N cells incubated in the serum-free experiment medium to exclude any effects of the serum. A very low level of Nurr1 mRNA expression (3.8 kb) was identified in unstimulated NTera2-N cells when incubated in the experiment medium without supplement of the serum (Fig. 3, left and right panels (a), lane 1). The levels of Nurr1 mRNA were elevated markedly in NTera2-N cells after an 1.5 or 3 h treatment with 100 nM PMA or 1 mM dbcAMP, when the levels of its expression were standardized against corresponding β-actin mRNA signals (1.8 kb) detected on the identical blots (Fig. 3, left

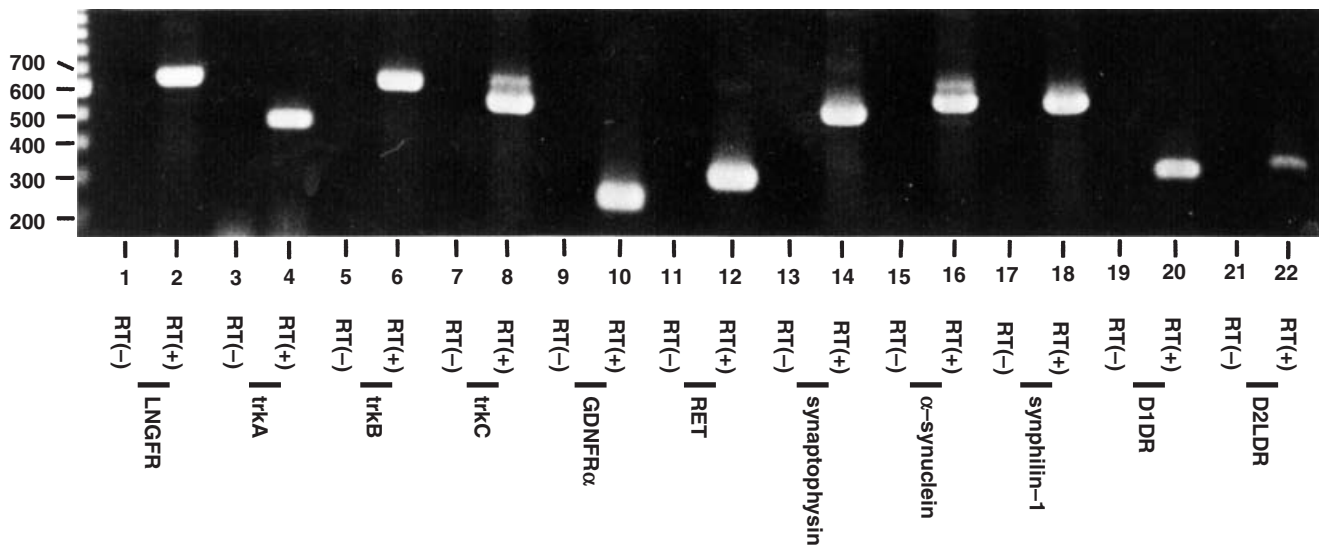

Fig. 2 Neurotrophic factor receptor mRNA expression in NTera2-N cells. Total RNA isolated from NTera2-N cells incubated in the feeding medium containing 10% FBS was processed for RT-PCR omitting the RT step (lanes 1,3,5,7,9,11,13,15,17,19,21) or following the RT step (lanes 2,4,6,8,10,12,14,16,18,20,22), using the primer sets listed in Table 1. The amplified products were separated on a 1.5% agarose gel and stained with ethidium bromide. Lanes (1–22) represent the following: (1,2) low-affinity NGF receptor (LNGFR) (3,4) the tyrosine kinase receptor trkA (5,6) trkB (7,8) trkC (9,10) GDNF receptor-α (GDNFRα) (11,12) the receptor tyrosine kinase RET (13,14) synaptophysin (15,16) α-synuclein (17,18) synphilin-1 (19,20) D1 dopamine receptor (D1DR), and (21,22) the long form of D2DR (D2LDR). The DNA size marker (bp) is shown on the left.

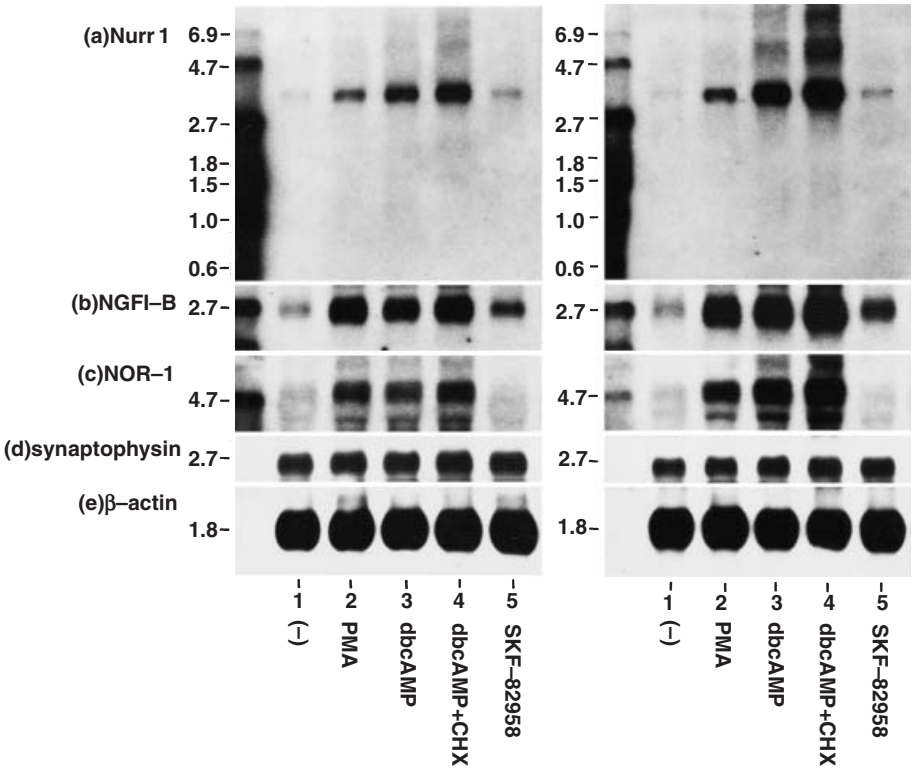

Fig. 3 Nurr1, NGFI-B and NOR-1 mRNA expression in NTera2-N cells after exposure to PKA and PKC activators. NTera2-N cells were incubated for 48 h in the serum-free experiment medium before initiation of the treatment. They were then incubated further for 1.5 h (left panel) or 3 h (right panel) in the serum-free experiment medium supplemented with PKA and PKC activators, followed by processing for RNA preparation. 3 μg of total RNA separated on a 1.5% agarose-6% formaldehyde gel was transferred onto a nylon membrane. It was then hybridized with the DIG-labeled DNA probe specific for Nurr1 gene (panel a), followed by re-hybridization with the probe specific for the genes encoding NGFI-B (panel b), NOR-1 (panel c), synaptophysin (panel d), or with the β-actin gene-specific RNA probe (panel e). Lanes (1–5) represent the following: (1) untreated (2) 100 nM phorbol 12-myristate 13-acetate (PMA) (3) 1 mM dibutyryl cyclic AMP (dbcAMP) (4) 1 mM dbcAMP plus 10 μg/mL cycloheximide (CHX), and (5) 200 mM SKF-82958 hydrobromide. The RNA size marker (kb) is shown on the left.

and right panels (a) and (e), lanes 2,3). However, they were reduced at 6 h after initiation of the treatment (data not shown). The inclusion of 10 μg/mL CHX in the experiment medium containing 1 mM dbcAMP greatly enhanced Nurr1 mRNA expression in NTera2-N cells, indicating the superinduction of Nurr1 mRNA where no de novo protein synthesis is required for its induction (Fig. 3, left and right panels (a), lane 4).[4,6] Treatment with SKF-82958 hydrobromide, a full D1DR agonist at a concentration of 200 μM, induced a discernible level of Nurr1 mRNA expression in NTera2-N cells (Fig. 3, left and right panels (a), lane 5), while a 1.5 or 3 h exposure to 100 ng/mL NGF, BDNF or GDNF did not significantly elevate Nurr1 mRNA levels (Fig. 4, left and right panels (a), lanes 1–4).

A low level of NGFI-B mRNA expression (2.8 kb) was identified in unstimulated NTera2-N cells incubated in the serum-free experiment medium (Fig. 3, left and right panels (b), lane 1). The levels of NGFI-B mRNA were elevated markedly in NTera2-N cells after a 1.5 or 3 h-treatment with 100 nM PMA or 1 mM dbcAMP, most robustly by treatment with 1 mM dbcAMP plus 10 μg/mL CHX, and moderately by exposure to 200 μM SKF-82958 hydrobromide (Fig. 3, left and right panels (b), lanes 2–5). Furthermore, the levels of NGFI-B mRNA were elevated substantially in NTera2-N cells by a 1.5 or 3 h treatment with 100 ng/mL BDNF, and to a smaller extent by NGF, but not induced by exposure to GDNF (Fig. 4, left and right panels (b), lanes 1–4).

NOR-1 mRNA expression was not found in unstimulated NTera2-N cells when incubated in the serum-free experiment medium (Fig. 3, left and right panels (c), lane 1). The levels of NOR-1 mRNA, composed of two distinct transcripts (4.0 kb and 5.5 kb), were elevated markedly in NTera2-N cells following a 1.5 or 3 h treatment with 100 nM PMA or 1 mM dbcAMP, most strongly induced by treatment with 1 mM dbcAMP plus 10 μg/mL CHX, but not by exposure to 200 μM SKF-82958 hydrobromide (Fig. 3, left and right panels (c), lanes 2–5). Again, NOR-1 mRNA expression was not found in NTera2-N cells after a 1.5 or 3 h treatment with 100 ng/mL NGF, BDNF or GDNF (Fig. 4, left and right panels (c), lanes 1–4). The levels of synaptophysin mRNA (2.6 kb) remained unchanged after exposure to PMA, dbcAMP, dbcAMP plus CHX, SKF-82958 hydrobromide, NGF, BDNF or GDNF (Figs. 3 and 4, left and right panels (d), lanes 1–5). These results indicate that Nurr1, NGFI-B and NOR-1 mRNA expression is coordinately up-regulated in NTera2-N cells following activation of the PKA or PKC pathway, while it is regulated differentially by treatment with NGF, BDNF or GDNF.

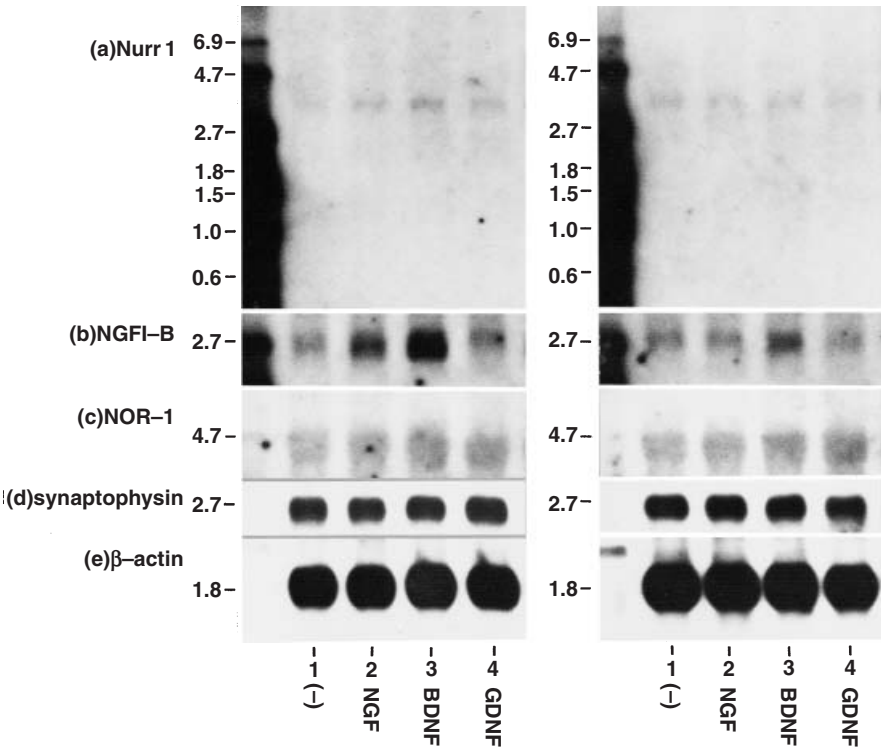

Fig. 4 Nurr1, NGFI-B and NOR-1 mRNA expression in NTera2-N cells after exposure to neurotrophic factors. NTera2-N cells were incubated for 48 h in the serum-free experiment medium before initiation of the treatment. They were then incubated further for 1.5 h (left panel) or 3 h (right panel) in the serum-free experiment medium supplemented with neurotrophic factors. 3 μg of total RNA separated on a 1.5% agarose-6% formaldehyde gel was transferred onto a nylon membrane. It was then hybridized with the DIG-labeled DNA probe specific for Nurr1 gene (panel a), followed by re-hybridization with the probe specific for the genes encoding NGFI-B (panel b), NOR-1 (panel c), synaptophysin (panel d), or with the β-actin gene-specific RNA probe (panel e). Lanes (1–4) represent the following: (1) untreated (2) 100 ng/mL NGF (3) 100 ng/mL BDNF, and (4) 100 ng/mL GDNF. The RNA size marker (kb) is shown on the left.

Overexpression of Nurr1 in HEK293 cells does not affect expression of 27 genes important for neuronal differentiation and survival

Finally, to identify the target genes whose expression is regulated by Nurr1, the expression vector containing either the ORF of Nurr1 gene or LacZ gene was transfected in HEK293, IMR-32, SK-N-SH, U-373MG or NTera2-U cells, and then incubated for 96 h in the serum-free experiment medium to exclude any effects of the serum. The expression of recombinant Nurr1 protein (70 kDa) or LacZ (120 kDa) was identified in HEK293 cells at 96 h after transfection (Fig. 5, left and right panels (a)-(c), lanes 2,4), with the transfection efficiency being over 80% determined by β-Gal staining of HEK293 cells transfected with the LacZ gene expression vector (data not shown). Neither Nurr1 mRNA nor Nurr1 protein were found to be expressed in non-transfected HEK293 cells under the serum-free culture condition (Fig. 5, left and right panels (a)-(c), lanes 1,3; Fig. 6a, lanes 1–3). By contrast, the expression of Nurr1 protein was under the detection limit in IMR-32, SK-N-SH, U-373MG or NTera2-U cells transfected with the Nurr1 gene expression vector (data not shown).

Twenty-seven candidates for Nurr1-target genes were selected in view of their potential involvement in neuronal growth, differentiation and survival.[29,31–38] By northern blot analysis, overexpression of either Nurr1 or LacZ in HEK293 cells incubated under the serum-free culture condition did not substantially change the levels of mRNA coding for NGFI-B (Fig. 6b, lanes 1–3), RA receptor-related orphan receptor alpha (RORα, 15 kb; Fig. 6e, lanes 1–3), BDNF (1.6 kb; Fig. 7a, lanes 1–3), LNGFR (3.8 kb; Fig. 7b, lanes 1–3), fibroblast growth factor receptor-1 (FGFR-1, 4.3 kb; Fig. 7c, lanes 1–3), GDNFRα (9.1 kb; data not shown), the proto-oncogene c-fos (2.2 kb; Fig. 7d, lanes 1–3), the proliferation-associated gene (PAG, 1.2 kb; Fig. 7e, lanes 1–3), α-synuclein (3.6 kb and 1.5 kb; Fig. 8a, lanes 1–3), defender against apoptotic cell death-1 (DAD1, 0.8 kb; Fig. 8b, lanes 1–3), cellular RA-binding protein-II (CRABP-II, 1.2 kb; Fig. 8c, lanes 1–3), 27-kDa heat shock protein (HSP27, 0.95 kb; Fig. 8d, lanes 1–3), or HSP70 (2.4 kb; Fig. 8e, lanes 1–3). Furthermore, the expression of mRNA encoding NOR-1 (Fig. 6c, lanes 1–3), RXRα (Fig. 6d, lanes 1–3), NGF, NT3, GDNF, trkA, trkB, trkC, RET, GDNFRβ, TH, synphilin-1, the growth-associated protein-43 (GAP-43), or the paired box gene-5 (Pax-5) was neither identified in non-transfected HEK293 cells nor induced in these cells after overexpression of Nurr1 (data not shown), although the NBRE-like sequences have been identified in promoter regions of TH, NGF, BDNF and CRABP-II genes.[15,27,28,39,40] These obser-

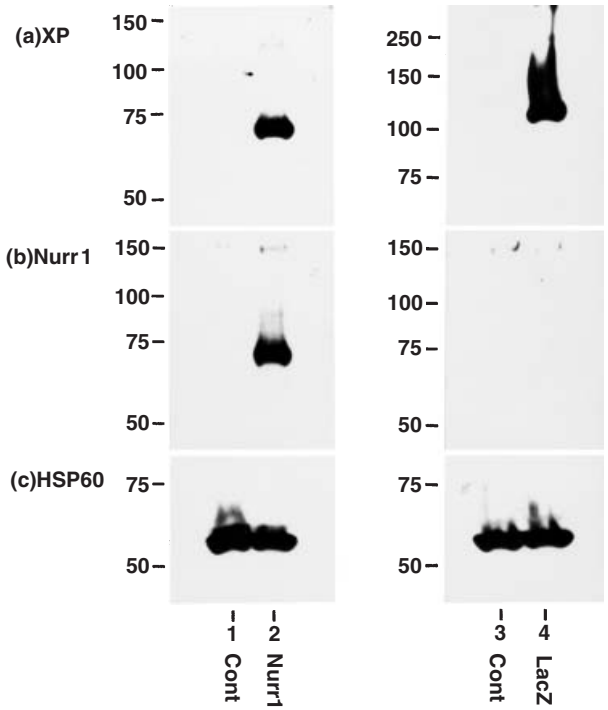

Fig. 5 Nurr1 protein expression in HEK293 cells transfected with the Nurr1 gene expression vector. The entire open reading flame (ORF) of Nurr1 gene was cloned in the vector pcDNA4/HisMax-TOPO which contains a N-terminal Xpress tag for detection of the recombinant protein. The vector containing either Nurr1 gene or LacZ gene was transfected in HEK293 cells incubated in the serum-free experiment medium for 24 h before transfection. After incubating the cells in the serum-free experiment medium further for 96 h, they were processed for protein preparation. 100 µg of total protein extract was separated on a 8% SDS-PAGE gel, transferred onto a nitrocellulose membrane, immunolabeled with anti-Xpress antibody (panel a), and processed for relabeling with anti-Nurr1 antibody (panel b) or with anti-HSP60 antibody (panel c). Lanes (1–4) represent the following: (1) non-transfected cells (2) the cells transfected with the Nurr1 gene expression vector (3) non-transfected cells, and (4) the cells transfected with the LacZ gene expression vector. The molecular weight marker (kDa) is shown on the left.

vations indicate that the induction of Nurr1 alone is insufficient for transcriptional activation of Nurr1 target genes in HEK293 cells.

DISCUSSION

The present study by using RT-PCR analysis has shown that Nurr1, NGFI-B and NOR-1 mRNA were coexpressed constitutively in various human neural and non-neural cell lines incubated in culture medium containing 10% FBS, a potent inducer for ONR expression.[3,4,41] These findings are apparently inconsistent with a previous study by northern blot analysis showing that Nurr1 mRNA is undetectable in both IMR-32 cells and HeLa cells under the serum-containing culture condition,[4] although this discrepancy is derived from the different sensitivities of the methods employed. Importantly, in spite of the widespread expression, targeted disruption of Nurr1 gene specifically inhibits terminal differentiation of midbrain dopaminergic neuronal precursor cells.[12–17] We found that the levels of Nurr1, NGFI-B and NOR-1 mRNA were elevated rapidly in NTera2-N cells, a model of differentiated human neurons, following exposure to dbcAMP or PMA without requirement of de novo protein synthesis, indicating that they represent immediate early-response genes.[4,6] We investigated the expression of 27 genes in HEK293 cells after overexpression of Nurr1, including the genes potentially involved in dopaminergic neuronal differentiation and survival. However, none of them showed significant alterations. Unexpectedly, HEK293 cells, a prototype of non-neural cell lines, expressed constitutively mRNA coding for α-synuclein, BDNF, LNGFR, FGFR-1 and GDNFRα, all of which play important roles in differentiation of dopaminergic neurons. These observations indicate that Nurr1, NGFI-B and NOR-1 mRNA expression is up-regulated in human neurons following activation of PKA or PKC pathway, and suggest that the induction of Nurr1 alone might be insufficient for transcriptional activation of Nurr1-target genes in HEK293 cells. Previous studies showed that treatment with forskolin or 12-*O*-tetradecanoylphorbol-13-acetate (TPA) elevates Nurr1, NGFI-B, and NOR-1 mRNA levels in human and rodent neural and non-neural cells,[5,6] supporting the present observations.

Recent studies showed that the promoter region of human Nurr1 gene has *cis*-acting regulatory elements activated through PKA or PKC pathways, such as a cyclic AMP-responsive element (CRE) and a consensus sequence for nuclear factor-kappaB (NF-κB).[42,43] Nurr1, NGFI-B and NOR-1 differ not only in the spatial and temporal distribution, but also in the inducibility in response to various stimuli.[6,9] NGFI-B was originally identified as a gene induced by NGF in the rat pheochromocytoma cell line PC12.[44] Unlike NGFI-B, Nurr1 is not induced in PC12 cells by NGF treatment, while the expression of both NGFI-B and Nurr1 is triggered by potassium-induced depolarization of PC12 cells.[1,44] We found that NGFI-B mRNA expression was up-regulated in NTera2-N cells preferentially by exposure to BDNF. BDNF elevates intracellular cyclic AMP levels in cultured cerebellar and hippocampal neurons,[45,46] suggesting a possible involvement of PKA pathway in BDNF-induced NGFI-B expression in NTera2-N cells, although a recent study indicated that BDNF stimulates c-fos mRNA transcription in cerebellar neurons through activation of CRE, but independently of the PKA pathway.[47] The CRE sequence is also identified

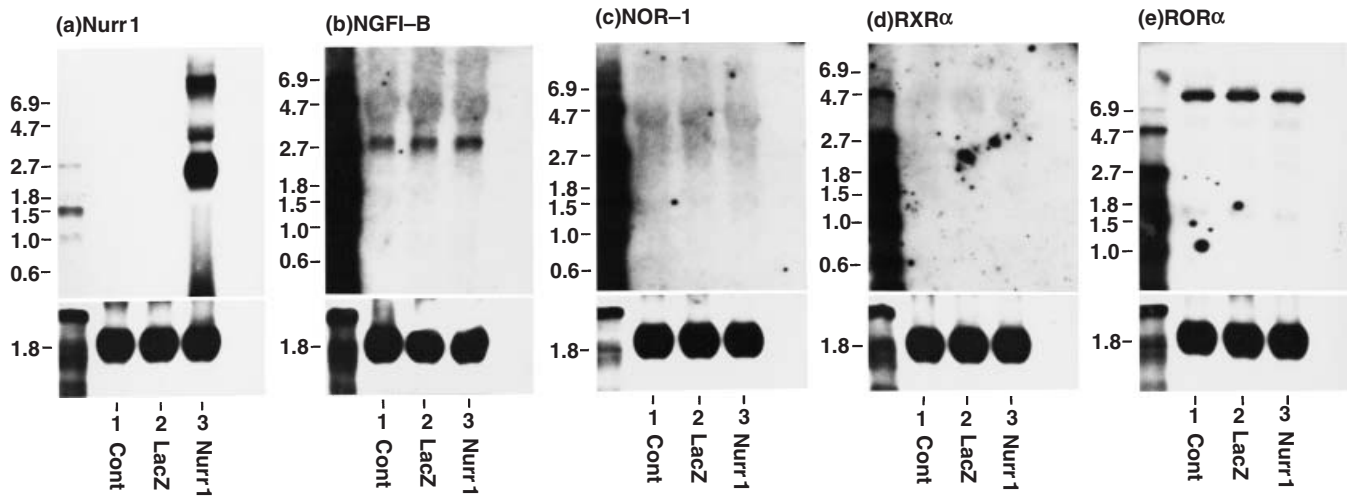

Fig. 6 Nurr1, NGFI-B, NOR-1, RXRα and RORα mRNA expression in Nurr1-overexpressing HEK293 cells. See the footnote of Fig. 5. Either the expression vector containing Nurr1 gene or LacZ gene was transfected in HEK293 cells incubated in the serum-free experiment medium for 24 h before transfection. After incubating the cells in the serum-free experiment medium further for 96 h, they were processed for RNA preparation. 3 μg of total RNA separated on a 1.5% agarose-6% formaldehyde gel was transferred onto a nylon membrane. It was then hybridized with the DIG-labeled DNA probe specific for the genes encoding Nurr1 (upper panel a), NGFI-B (upper panel b), NOR-1 (upper panel c), retinoid X receptor alpha (RXRα) (upper panel d), or RA receptor-related orphan receptor alpha (RORα) (upper panel e). The blots were re-hybridized with the β-actin gene-specific RNA probe (lower panels). Lanes (1–3) represent the following: (1) non-transfected cells (2) the cells transfected with the LacZ gene expression vector, and (3) the cells transfected with the Nurr1 gene expression vector. The RNA size marker (kb) is shown on the left.

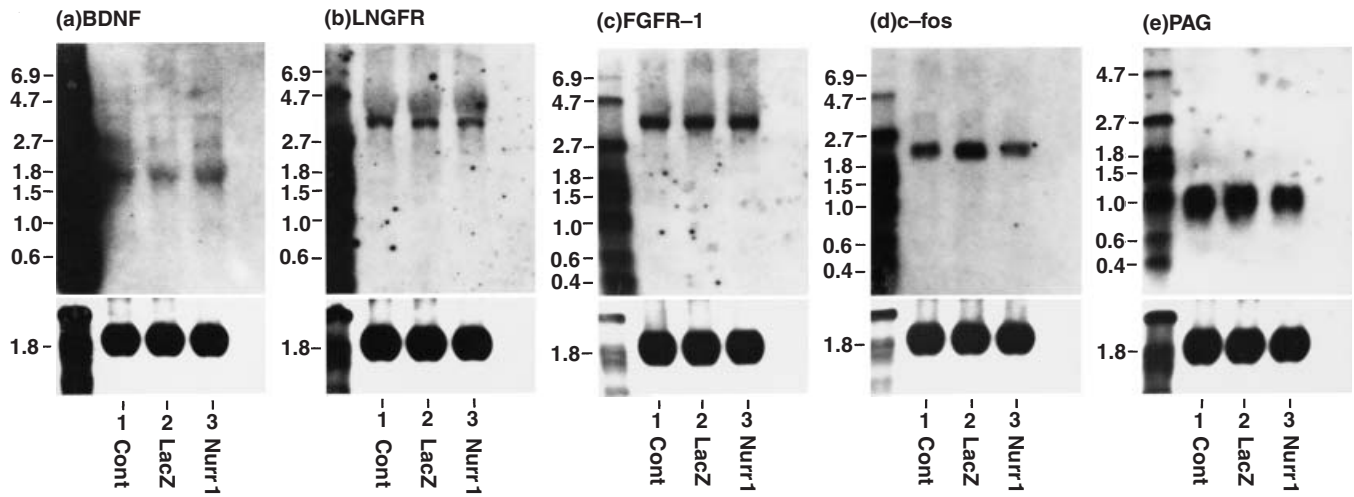

Fig. 7 BDNF, LNGFR, FGFR-1, c-fos and PAG mRNA expression in Nurr1-overexpressing HEK293 cells. See the footnote of Fig. 6. 3 μg of total RNA separated on a 1.5% agarose-6% formaldehyde gel was transferred onto a nylon membrane. It was then hybridized with the DIG-labeled DNA probe specific for the genes encoding BDNF (upper panel a), LNGFR (upper panel b), fibroblast growth factor receptor-1 (FGFR-1) (upper panel c), protooncogene c-fos (upper panel d), or proliferation-associated gene (PAG) (upper panel e). The blots were re-hybridized with the β-actin gene-specific RNA probe (lower panels). Lanes (1–3) represent the following: (1) non-transfected cells (2) the cells transfected with the LacZ gene expression vector, and (3) the cells transfected with the Nurr1 gene expression vector. The RNA size marker (kb) is shown on the left.

in promoter regions of human NGFI-B and NOR-1 genes.[48,49]

A biological role of Nurr1 in neuronal function in the human CNS remains totally unknown. Nurr1 mRNA expression is induced in rat hippocampal CA1 and CA3 pyramidal neurons, closely associated with acquisition of long-term spatial memory.[50] Its up-regulation is also observed in rat hippocampal neurons following intracerebral injection of ibotenic acid.[51] Both Nurr1 and NGFI-B mRNA levels are elevated in rat cerebral cortical neurons

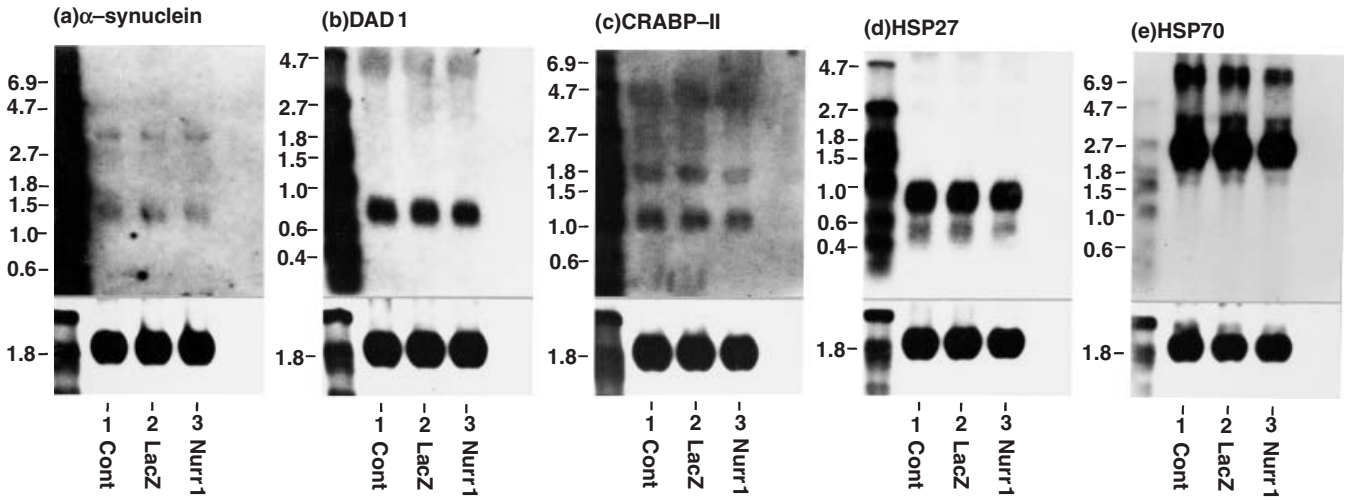

Fig. 8 α-Synuclein, DAD1, CRABP-II, HSP27 and HSP70 mRNA expression in Nurr1-overexpressing HEK293 cells. See the legend to Fig. 6. 3 μg of total RNA separated on a 1.5% agarose-6% formaldehyde gel was transferred onto a nylon membrane. It was then hybridized with the DIG-labeled DNA probe specific for the genes encoding α-synuclein (upper panel a), defender against apoptotic cell death-1 (DAD1) (upper panel b), cellular RA-binding protein-II (CRABP-II) (upper panel c), 27-kDa heat shock protein (HSP27) (upper panel d), or HSP70 (upper panel e). The blots were re-hybridized with the β-actin gene-specific RNA probe (lower panels). Lanes (1–3) represent the following: (1) non-transfected cells (2) the cells transfected with the LacZ gene expression vector, and (3) the cells transfected with the Nurr1 gene expression vector. The RNA size marker (kb) is shown on the left.

following cerebral ischemia.[52] Nurr1 mRNA expression is up-regulated in neurons of rat hippocampal dentate gyrus following kainic acid-induced and electroconvulsive seizures.[53,54] These observations suggest that diverse neuronal activity-dependent stimuli promptly induce Nurr1 expression in defined populations of neurons. Nurr1, NGFI-B and NOR-1 are also found to be expressed abundantly in tissues of the hypothalamus-pituitary-adrenal (HPA) axis, where both Nurr1 and NGFI-B activate the transcription of corticotropin-releasing hormone (CRH) and proopiomelanocortin (POMC) genes, suggesting a fundamental role of ONR in regulation of neuroendocrine function.[55–58]

The NBRE-like sequences have been identified in promoter regions of the genes encoding CRH, POMC, steroid 21-hydroxylase, cholesterol desmolase, DAT, TH, NGF, BDNF, vasopressin, oxytocin, CRABP-II, apolipoproteins C-III and B-100, prealbumin, acyl-coenzyme A oxidase, alkaline phosphatase, bone sialoprotein, osteocalcin, type I collagen, collagenase, insulin-like growth factor I, acetylcholine receptor and N-myc.[15,27,28,39,40,58–60] Furthermore, previous studies suggest that the expression of both GDNFRβ and RET genes is regulated by Nurr1 in mouse neural cells.[18,61] However, we found that the expression of mRNA encoding TH, NGF, BDNF, GDNFRβ, RET or CRABP-II was not affected by overexpression of Nurr1 in HEK293 cells. One possible explanation for this discrepancy is that HEK293 cells might lack auxiliary proteins acting as a coactivator for transcription of Nurr1-target genes in dopaminergic neuronal precursor cells.[21,22,24] The expression of Nurr1 protein was not identified in IMR-32, SK-N-SH, U-373MG or NTera2-U cells after transfection of the expression vector, hampering the use of neural cell lines as a preferable source to identify Nurr1-target genes. The lack of Nurr1 expression in these cell lines is attributable either to the lower transfection efficiency or to the reduced activity of promoter elements of the vector in certain cell types.

Another possibility could be proposed that HEK293 cells which do not express RXRα, a heterodimeric partner for Nurr1 and NGFI-B,[9,23,24] are not fully competent for transcriptional activation of Nurr1-target genes. However, a recent study indicates that Nurr1, without formation of a heterodimer with RXR, promotes differentiation of dopaminergic MN9D cells.[62] Nurr1, NGFI-B, and NOR-1 exhibit different selectivities for the NBRE-responsive genes and the LBD of Nurr1 influences the ability to activate transcription of the target genes, depending on cell type and mode of DNA binding.[21,63] Nurr1 activates transcription of the human DAT gene in a mouse dopaminergic neuronal cell line by acting as a coactivator of an undefined protein.[64] By contrast, Nurr1 represses transcription of the glutamate receptor ionotropic kainate 5 (GIRK5) gene by binding to a small intronic element.[65] Furthermore, post-translational modifications of Nurr1 protein might affect transcriptional activation of the target genes.[4,56] All of these observations indicate that the mechanisms underlying Nurr1-mediated transcriptional regula-

tion are highly complex, involving NBRE-independent mechanisms.

In conclusion, Nurr1 mRNA expression is up-regulated in differentiated human neurons following activation of PKA or PKC pathway, suggesting that diverse extracellular stimuli activate transcription of Nurr1 in human neurons, although its target genes remain to be identified.

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