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Biopterin metabolism in patients with malignant syndrome

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Abstract

We examined the autopsied brains of two parkinsonian patients who had malignant syndrome (MS). Neopterin and biopterin contents, and GTP cyclohydrolase I activity were measured in various region of the brain. We found relatively higher GTP cyclohydrolase I activities in the hypothalamus compared with other regions of the brain from patients with MS. This finding suggested a possible involvement of biopterin metabolism in pathophysiology of MS. This is the first report on biopterin metabolism in the brains of patients with MS.

Keywords: Dopamine; Tetrahydrobiopterin; Neopterin; GTP Cyclohydrolase I

Dopaminergic drugs, such as L-dopa, bromocriptine, and amantadine, are widely used for the treatment of Parkinson's disease (PD) It has been reported that symptoms called 'malignant syndrome' (MS) occur in a small group of parkinsonian patients following abrupt withdrawal of dopaminergic drugs [1–3]. The symptoms are similar to those seen in a group of patients administered neuroleptic drugs, which act as dopamine blockers. The latter is called neuroleptic MS. Although the pathophysiology of MS is unknown, much circumstantial evidence suggests the possible involvement of the brain dopamine system.

Tetrahydrobiopterin is an essential cofactor for aromatic amino acid hydroxylases, tyrosine hydroxylase, tryptophan hydroxylase, and phenylalanine hydroxylase. Tetrahydrobiopterin is synthesized from guanosine triphosphate (GTP) through three enzymatic reactions. GTP cyclohydrolase I is the enzyme that catalyzes the first and rate-limiting step of biopterin biosynthesis, the formation of D-erythro-dihydroneopterin triphosphate from GTP [4].

We found that hereditary progressive dystonia with marked diurnal fluctuation (HPD) [5], also known as dopa-responsive dystonia (DRD) [6], is caused by a genetic defect in the GTP cyclohydrolase I gene [7]. HPD/DRD is a disorder typically characterized by concurrent or subsequent development of parkinsonism and a dramatic therapeutic response to L-dopa. Our finding suggested the importance of biopterin-metabolism in parkinsonism and related disorders.

In order to explore pathophysiology of MS, we analyzed the GTP cyclohydrolase I activity, and the neopterin and biopterin contents in the autopsy human brain from controls and parkinsonian patients who had fatal MS.

1. Materials and methods

Control human brains and parkinsonian brains were obtained from brain banks in Japan and stored frozen at −70 °C. The activity of GTP cyclohydrolase I was determined as described previously with a slight modification [7]. The brain tissues were homogenized in 4 vol of cold 50 mM Tris–HCl buffer (pH 7.5) containing 10% glycerol and 1 mM dithiothreitol. The homogenate was used as an enzyme source. The reaction mixture (100 μl) contained 1 mM GTP, 2.5 mM EDTA, 0.3 M KCl, 10% glycerol in 0.1 M Tris–HCl buffer (pH 8.0), and 20 μl of the enzyme solution. After incubation at 37 °C for 60 min in the dark, the product, dihydroneopterin–triphosphate, was first oxidized with iodine and was then dephosphorylated by alkaline phosphatase; neopterin formed was assayed by HPLC with fluorescence detection. Total biopterin derived from L-erythro-tetrahydrobiopterin and total neopterin derived from D-erythro-7,8-dihydroneopterin triphosphate

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Table 1
Case histories of control and parkinsonian patients

Case	Sex/age	Neurological disorder	Cause of death	Post-mortem period (h)
Control				
No. 92	M/80	None	Heart failure	3
No. 93	M/80	None	Heart failure	0.5
No. 95	M/77	None	Pneumonia	1
Patients				
Case 1	F/65	PD	Stomach cancer	6
Case 2	F/62	PD	MS	4
Case 3	F/65	Juvenile PD	MS	4.5

were determined by HPLC with fluorescence detection. Protein was assayed by the Bradford method [8].

2. Results and discussion

Data on all individuals examined are summarized in Table 1.

GTP cyclohydrolase I activities and neopterin and biopterin contents in the various regions of the brain in 3 control cases, 1 case of PD, 1 case of PD who died of MS, and 1 case of juvenile PD who died of MS are shown in Table 2. GTP cyclohydrolase I activity was detectable in the autopsied human brain examined by the sensitive HPLC assay. Even though not reaching statistical significance, GTP cyclohydrolase I activities and neopterin and biopterin levels in the putamen of 2 cases of PD showed a tendency toward decrease especially as compared with those in control No. 92. This is in accordance with a previous report on GTP cyclohydrolase I with more parkinsonian brains [9]. In contrast, the ratio of neopterin/biopterin showed a trend towards increase in PD patients, in accordance with our previous report on that in cerebrospinal fluid from PD patients [10].

The most striking difference between the patient with MS and the one without the syndrome was distribution of GTP cyclohydrolase I activity (Fig. 1). Patients with MS (cases 2 and 3) showed the highest activity in the hypothalamus compared with the activity in their other regions of the brain, while the GTP cyclohydrolase I activity in the hypothalamus of the patient without MS was similar to that in the substantia nigra in the patient without the syndrome. Higher levels of biopterin were also found in the hypothalamus in patients with MS (cases 2 and 3) compared with other regions of the brain (Table 2). The highest biopterin value was observed in the hypothalamus in MS patients, but the biopterin level of the PD patient without MS (case 1) was highest in the substantia nigra.

In a study using autopsy human brain, it is sometimes difficult to compare the value of each case because an

Table 2
GTP cyclohydrolase I activity, and neopterin and biopterin contents in the brain from control and parkinsonian patients

	GTP cyclohydrolase I (pmol/h/mg protein)	Neopterin (pmol/mg protein)	Biopterin (pmol/mg protein)	Neo/bio
Putamen				
Control (No. 92)	1.22	0.87	11.15	0.08
Control (No. 93)	0.62	1.00	4.29	0.23
Control (No. 95)	0.51	0.50	2.71	0.18
PD (case 1)	0.81	0.79	3.18	0.25
PD-MS (case 2)	0.55	2.92	4.53	0.64
JP-MS (case 3)	0.28	0.72	2.64	0.27
Substantia nigra				
PD (case 1)	4.63	0.85	18.29	0.05
PD-MS (case 2)	2.11	2.12	12.20	0.17
JP-MS (case 3)	1.00	0.64	7.04	0.09
Pallidum				
PD (case 1)	1.52	0.77	7.42	0.10
PD-MS (case 2)	0.97	2.03	8.76	0.23
JP-MS (case 3)	0.35	0.77	3.75	0.21
Hypothalamus				
PD (case 1)	4.44	0.39	7.61	0.05
PD-MS (case 2)	5.60	1.42	18.95	0.07
JP-MS (case 3)	4.03	0.57	7.85	0.07

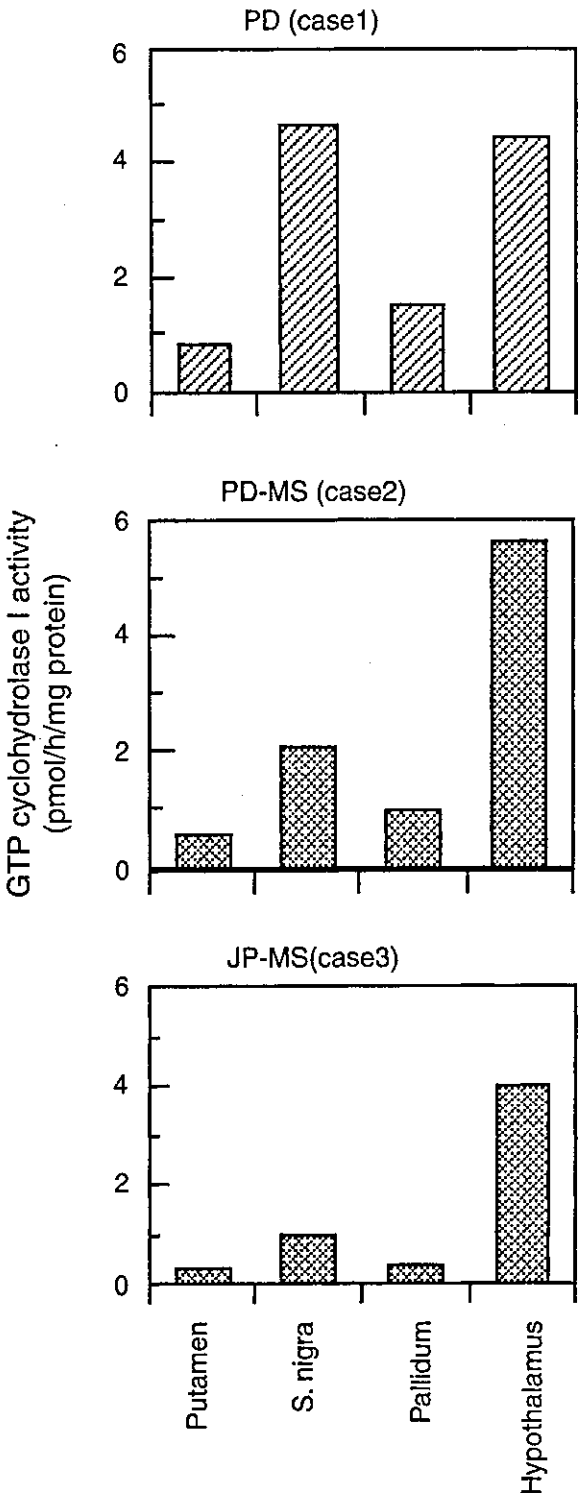

Fig. 1. Distribution of the GTP cyclohydrolase I activity in the putamen, substantia nigra, pallidum, and hypothalamus of parkinsoninan patients.

enzyme's activity is affected by the time of the examination post-mortem and individual variations. However, it has been assumed that the distribution of enzyme activity is not dependent on the post-mortem time and individual variation. Our results suggested that biopterin metabolism was enhanced in the hypothalamus in patients with MS, although this needs to be confirmed with a larger number of samples.

We can see a good correlation between GTP cyclohydrolase I activity and biopterin content. However, neopterin content did not correlate well with GTP cyclohydrolase I activity. This may suggest that neopterin content may not be totally dependent on GTP cyclohydrolase I activity and that other factors may affect the cellular content of neopterin.

Out of three parkinsonian patients, case 1 who suffered from juvenile PD showed lowest GTP cyclohydrolase I activity in all regions examined, especially in substantia nigra (Table 2). This suggests that the altered biopterin metabolism may participate in the pathogenesis for a type of juvenile PD.

There are important neurons in the hypothalamus that regulate functions of autonomic nervous system. It is thought that hyperactivity of the autonomic nervous system causes the main symptoms of MS, that is, hyperpyrexia, sweating, and tachycardia. We found that biopterin synthesis in the hypothalamus was increased in patients with MS compared with other regions of the brain. Since tetrahydrobiopterin is an essential cofactor for tyrosine hydroxylase, the rate-limiting enzyme for catecholamine synthesis [11], the increased biopterin-synthesis may mean hyperactivity of noradrenergic neurons in the hypothalamus.

The levels of noradrenaline and its major metabolite, 3-methoxy-4-hydroxyphenylethyleneglycol, have been reported to increase significantly during the active phase and to return to normal after recovery in the cerebrospinal fluid of patients with NMS [12]. In 1990, Kish et al. [13] reported marked hypothalamic noradrenaline depletion in three patients who had a fatal hyperthermia syndrome. This report may also support hyperactivity of noradrenergic neurons in the hypothalamus, since depletion of noradrenaline seems to be caused by increased rate of depolarization.

Neuroleptic MS is caused by administration of dopamine-blocking drugs, and similar symptoms are sometimes observed in patients with PD soon after they stop taking dopaminergic drugs. Thus, a sudden blockade of the dopaminergic system may lead to the appearance of symptoms of MS. The abnormally low functional activity of the brain dopamine system in MS would be the reason for the successful treatment of patients with dopaminergic agonists, such as bromocriptine and dantrolene [14].

Gurrera proposed an hypothesis that dysregulated sympathetic nervous system hyperactivity is the pathophysiological basis of neuroleptic MS [15]. Since the sympathetic nervous system is regulated by the hypothalamus, the increased biopterin metabolism in the hypothalamus could be involved in pathogenesis of MS.

There are two possible explanations on the alteration. One explanation is that the increased noradrenaline and/or serotonin synthesis by the enhanced biopterin metabolism caused the dysregulation of the sympathetic nervous system. The other is that the biopterin synthesis was enhanced in

order to produce more amount of dopamine in the hypothalamus and to inhibit the dysregulated sympathetic neurons by way of the inhibitory dopaminergic hypothalamospinal tracts. At present, we cannot determine the reason for the enhanced biopterin metabolism. Further studies with more cases of MS are required to clarify this.

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Sonic hedgehog and FGF8 collaborate to induce dopaminergic phenotypes in the Nurr1-overexpressing neural stem cell

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Abstract

Neural stem cells are self-renewing cells capable of differentiating into all neural lineage cells in vivo and in vitro. In the present study, coordinated induction of midbrain dopaminergic phenotypes in an immortalized multipotent neural stem cell line can be achieved by both overexpression of nuclear receptor Nurr1, and fibroblast growth factor-8 (FGF-8), and sonic hedgehog (Shh) signals. Nurr1 overexpression induces neuronal differentiation and confers competence to respond to extrinsic signals such as Shh and FGF-8 that induce dopaminergic fate in a mouse neural stem cell line. Our findings suggest that immortalized NSCs can serve as an excellent model for understanding mechanisms that regulate specification of ventral midbrain DA neurons and as an unlimited source of DA progenitors for treating Parkinson disease patients by cell replacement.

Keywords: Nurr1; Dopaminergic neurogenesis; Tyrosine hydroxylase; Sonic hedgehog; FGF8; Human fetal astrocyte; Parkinson's disease

Dopaminergic (DA) neurons that lie in the ventral midbrain play a key role in voluntary movement, emotional behavior, and cognition. Loss of these neurons is associated with Parkinson's disease [1,2]. Recent advances in neural stem cell (NSC) biology have shown that multipotent neural progenitors can be isolated, expanded, and used as source material for brain transplants [3–5]. However, although multiple studies demonstrate that implanted neural progenitors successfully engraft and assume legitimate neural phenotypes, when transplanted in the intact adult brain, these cells seem biased toward astroglial and oligodendroglial fates [6–8]. Given that most neurodegenerative diseases affect neuronal populations of a specific neurochemical phenotype, an ideal source material for transplantation would be an expandable cell that could be instructed to completely assume the desired neuronal phenotype upon differentiation. However, the complete and coordinated induction of a specific neuronal phenotype in multipotent neural precursors in vitro has proved elusive. Our goal in this study was to define the factors required by NSCs to produce a DA phenotype, the major cell type lost in human Parkinson's disease.

One such factor is Nurr1, a transcription factor of the thyroid hormone/retinoic acid nuclear receptor superfamily [9]. Nurr1 is specifically required for the induction of midbrain DA neurons, which fail to develop in Nurr1-null mutant mice [10–12]. It is unclear, however, whether Nurr1 is also sufficient to specify this neurochemical phenotype. To test this phenotype, we stably overexpressed Nurr1 in an immortalized mouse NSC line termed A3, which does not normally give rise to DA neurons in vitro [13]. These cells possess the ability to differentiate into neurons, astrocytes, and oligodendrocytes in vitro and in vivo, and upon transplantation into the developing brain, adopt regionally appropriate

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neuronal phenotypes. We hypothesized that the NSC cell line, A3, fulfills all necessary criteria to identify the factors sufficient to reconstitute DA neuron development in vitro and to generate renewable source material for transplantation.

Cells in the fetal ventral mesencephalon express neurotrophic factors for DA neurons [14]. In addition, induction of mesencephalic DA neurons is mediated by the concerted action of diffusible and contact-dependent signals from the ventral mesencephalon [15–17]. These findings have suggested that non-neural cells in fetal ventral mesencephalon play an important role in generating and maintaining DA neurons in vivo and have prompted their use to increase the number of DA neurons from various progenitors. Similarly, it has recently been suggested that differentiation of DA neurons in vivo is dependent on critical cooperation of two signals. Implicated in these studies [15] are one member of the fibroblast growth factor (FGF) family, FGF8 [18], and the cell patterning molecule first described in *Drosophila*, sonic hedgehog (Shh) [19]. It is believed that developing cells differentiate into DA neurons when they encounter intersecting signals occurring along the anterior–posterior (FGF8) and dorsal–ventral (Shh) axes [15].

Materials and methods

Culture of mouse NSCs in culture. Mouse NSC line A3 was established as described previously [13], and maintained and passaged on uncoated culture dishes in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum or in DMEM supplemented with N2 supplement (Gibco-BRL, Gaithersburg, MD) to which was added to bFGF (10 ng/ml) (R&D Systems, Minneapolis, MN) + heparin (10 ng/ml) (Sigma, St. Louis, MO) (N2 plus bFGF medium). Medium was changed every 5 days. Cells were transferred to media containing serum for transduction of retroviral vector carrying Nurr1 gene.

Retrovirus-mediated gene transfer. Amphotropic replication-incompetent retroviral vector was used to infect the mouse NSC line. A vector encoding human Nurr1 gene was generated using the retroviral vector pBabe-puro to infect the PA317 amphotropic packaging cell line. Mouse NSC line was infected with a retrovirus encoding human Nurr1 gene. Mock controls received retrovirus harboring only pBabe-puro vector.

Cloning of NSCs overexpressing human Nurr1 gene. Cells were dissociated, diluted to 1 cell/100 μl, and plated at 100 μl/well of a 96-well plate. Wells with single cells were immediately identified. Single-cell clones were expanded and maintained in bFGF-containing growth medium. A total of 28 stable Nurr1 overexpressing clones were selected for puromycin resistance and isolated. The expression of Nurr1 mRNA was determined by Northern blot analysis in each clone. A 350 bp antisense RNA probe spanning nucleotides 1436–1786 of the human Nurr1 cDNA sequence was transcribed with SP6 and labeled with [^{32}P]CTP (NEN, London, UK).

RT-PCR and semi-quantitative PCR. Total RNA was isolated from cell cultures using RNAsol (Tel-Test, Friendswood, TX) and cDNA was made using the Superscript II reverse transcriptase (Gibco-BRL) from 4 μg of total RNA. First strand cDNA was diluted 3-fold and 2 μl of cDNA was used for each PCR. RT-PCR products were analyzed in an agarose gel containing ethidium bromide. DNA bands were photographed using a Gel Doc 2000 video system (Bio-Rad, Richmond, CA). The image was exported in a TIFF file and DNA bands were quantified using Quantity One 4.0.3 software. The quantification value of the band was designated by the absorbance (Pixels). Measurements of absorbance using the above system were linear up to 120 pixels. For quantitative PCR, cycle number and template quantity were determined to be in the linear range for each gene. Three independent PCRs were done for each experiment. For each condition, at least two independent experiments were carried out.

The following primers were used to amplify target cDNA.

(1) GAPDH	5′-GCAGGGGGGAGCCAAAAGGG-3′,
	5′-TGCCAGCCCCAGCGTCAAAG-3′
(2) TH	5′-AAGGTCCCCTGGTTCCCAAGAAAAGT-3′,
	5′-TCCTCCAGCTGGGGGATATTGTCTTC-3′
(3) AADC	5′-CCTGCTGGCCGCTCGGACCAA-3′,
	5′-GCGCACCAGTGACTCAAACTC-3′
(4) DBH	5′-GTGACCAGAAGGGGCAGATCC-3′,
	5′-GGCCGGCTTCCTCTGGGTAGT-3′

Treatment of neurotrophic factors and co-culture with astrocytes. Mouse NSC line was plated at a final density of 2×10^4 cells/cm^2 on poly-D-lysine- and laminin-coated culture wells in N2 plus bFGF medium to investigate the effects of various neurotrophic factors. After 24 h, all factors were added with fresh medium. Half of medium was replaced every 3 days with fresh medium containing factors.

For co-culture of NSCs and astrocytes, $2.5–5 \times 10^4$ human fetal astrocytes were seeded in laminin-coated insert (Millipore, Bedford, MA) with a membrane with 0.4 μm pores (sufficient to allow passages of proteins but not cells). Purified human fetal astrocytes were obtained from mixed glial cell cultures derived from human fetal brains according to a standard protocol. After 1 day, the membrane inserts was immersed in a well at the bottom of which rested the coverslips. All ages of cultures given use this point as day zero. After 8–10 days, coverslips were fixed and examined for TH gene expression. Half of medium was replaced every 3 days with fresh medium.

NSC cell line overexpressing Shh-N proteins. Mouse NSC line was infected with LPC-Shh-N retrovirus in 4 μg/ml DEAE–dextran for 4 h. Two days after infection, cells were selected for puromycin (5 μg/ml) resistance for 10 days. A pooled population of puromycin-resistant cells was analyzed for expression of Shh-N proteins and used in co-culture to treat mouse NSC line with Shh-N. Intracellular and secreted Shh-N proteins were evaluated by Western blot analysis and Sandwich ELISA technique, respectively. To determine the intracellular Shh protein level, cells were lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% NaDOC, 0.1% SDS, and 50 mM Tris, pH 8.0). Cell lysates were fractionated by SDS–PAGE. The immunoblotting procedure was done with ECL Western blot detection reagents (Amersham, Little Chalfont, UK). To examine secreted Shh-N proteins, 96-well Microtest assay plate (Becton–Dickinson, San Jose, CA) was coated with 5 μg of anti-Shh antibody (Santa Cruz Biotech, CA) overnight at 4 °C per well. Plate was incubated with conditioned media from A3 or A3.Shh-N cells, followed by incubation with 5E1 monoclonal anti-Shh antibody. Bound antibodies were detected by alkaline phosphatase-conjugated anti-mouse IgG antibody. After washing, 100 μl of 0.25 mM *p*-nitrophenyl phosphate disodium salt and 0.5 mM magnesium chloride in 10 mM diethanolamine buffer (pH 9.5) was added. Absorbance was determined at 405 nm and the ELISA cut-off value was defined as two times the optical density (OD) value of the conditioned medium from A3 cells.

Treatment with FGF-8 and Shh-N. Shh-N-overexpressing mouse NSC line ($2.5–5 \times 10^4$) was seeded on laminin-coated side of a membrane with 0.4 μm pores. Target mouse NSCs were plated at a final density of 2×10^4 cells/cm^2 on poly-D-lysine- and laminin-coated culture wells in N2 plus bFGF medium. After 24 h, the membrane inserts were immersed in a well at the bottom of which rested the coverslip in the presence or absence of murine FGF8 isoform b (R&D Systems). All ages of cultures given use this point as day zero. After 7 days, coverslips were processed for RT-PCR or immunocytochemistry for DA neuron related gene expression.

Immunocytochemical analysis. Cells were fixed with 4% paraformaldehyde for 20 min at room temperature before immunocytochemical staining. Fixed cultures were incubated with one of the following antibodies, diluted in PBS containing 1% BSA and 0.3% Triton X-100:mouse anti-tubulin β III (TuJ1, 1:250, Sigma, St. Louis, MO), or sheep polyclonal anti-TH (1:500, Pel-freeze, Rogers, AR). Incubations were carried out at 4 °C overnight. After washing, cultures were incubated with biotinylated horse anti-mouse IgG or goat anti-sheep IgG (1:100, Vector, Burlingame, CA) for 1 h and then reaction products were visualized with AEC substrates (Vector, Burlingame, CA).

Results

Nurr1 expression induces neuronal differentiation in mouse NSCs

Mouse NSC line A3 [13] was stably transduced with retrovirus encoding pBabe-Nurr1, and 28 clones expressing Nurr1 were analyzed for transgene expression by Northern blotting (data not shown). Several clones overexpressed Nurr1 gene, of which the highest expressor was chosen and designated A3.Nurr1 for further experiments. A3.Nurr1 cells behaved similar to the parental and pBabe-transduced clones, with exceptions that they showed slightly slow growth rate and sensitivity to cell–cell contact. To define developmental potential and phenotypic expression of Nurr1 clones, we examined their behavior following growth in serum-free defined medium (SFM) and serum containing medium. In A3.Nurr1 cells, many filopodia around cell bodies were induced compared to parental cell line A3 in serum containing medium (Fig. 1). This morphology could be clearly visualized by staining for actin and paxillin (Figs. 1C–F). Approximately 80% of A3.Nurr1 adopt a neuronal fate, judged by the expression of tubulin β III, a phenomenon not seen in any of the mock clones (data not shown). However, none of A3.Nurr1 cells expressed tyrosine hydroxylase (TH, a marker for DA neurons) under these conditions, suggesting that Nurr1 alone is not sufficient to induce DA phenotypes in mouse NSCs (data not shown).

Characterization of inductive signal

As Nurr1 possessed lineage-restricting capacity in our NSC line but alone is not sufficient to induce DA phenotypes, A3.Nurr1 cell line was treated with agents known to be important in DA neuronal differentiation. Since retinoids and forskolin have been previously implicated in promoting dopamine biosynthesis in primary central nervous system (CNS) progenitor cell cultures [20–22], the effects of 9-*cis*-retinoic acid (RA) and forskolin (FK) on induction of DA phenotypes [expression of TH, AADC, and dopamine-β-hydroxylase (DBH)] in A3.Nurr1 cells were investigated. A small number of Nurr1-overexpressing NSCs were labeled for TH ($4.9 \pm 0.3\%$ in the presence of RA and $3.5 \pm 0.4\%$ in the presence of FK) (Fig. 2B). In addition, some cells were immunoreactive for AADC but none contained detectable DBH (not shown). Consistent with the immunocytochemistry results, RT-PCR showed increased expression of TH and AADC, but absence of detectable DBH mRNA (Fig. 2A). Taken together, these results indicate that although minor in proportion, TH-immunoreactive cells generated in A3.Nurr1 cells are dopaminergic rather than adrenergic or nonadrenergic neurons.

Co-culture with human fetal astrocytes

None of retinoic acid or forskolin, alone or combined (data not shown), induced significant TH expression in any of the Nurr1 clones. To test whether as yet unidentified, regionally specific local factors were required, we co-cultured the Nurr1 clones with primary astrocyte cells derived from human fetal brain using membrane insert. Under these conditions a small, yet significant, percentage of isolated cells ($24.7 \pm 2.3\%$) and mitotic clusters, frequently shown, from the Nurr1 line demonstrated measurable amounts of TH immunoreactivity (Fig. 3C), whereas little or no TH staining was seen in the parental, or any of the mock control lines, indicating that human fetal astrocytes produce a specific activity that acts on Nurr1-overexpressing NSCs. Addition of RA and FK slightly increased TH-immunoreactive cell number in co-culture with human fetal astrocyte cells ($25.5 \pm 1.7\%$ for addition of FK to co-culture; $30.1 \pm 2.3\%$ for addition of FK and RK) (Fig. 3C). This activity from astrocytes might be very labile and diffusible factors, because it could be recovered not from conditioned media, but from co-cultures separated by a microporous insert of human fetal astrocytes (data not shown).

In A3.Nurr1 cells co-cultured with fetal astrocytes, TH mRNA was expressed at 5–10-fold higher levels than in control A3.Nurr1 cells. Expression of AADC mRNA was elevated 55-fold in co-cultured A3.Nurr1 cells and 95-fold in co-cultured A3.Nurr1 cells with FK and RA (Figs. 3A and B). Collectively, these observations suggested that a factor derived from the primary astrocyte cultures interacted directly or indirectly with Nurr1 to induce TH-expressing neurons.

Isolation of immortalized NSCs expressing Shh-N transgene

Since Shh and FGF-8 have been previously shown to promote ventral midbrain fates in neural plate explant [15], we hypothesized that the addition of these factors to NSCs might also increase the proportion of DA neurons in our culture system. To identify if signals that provide positional information for the developing DA neurons do work in mouse NSCs, we have first produced Shh-N

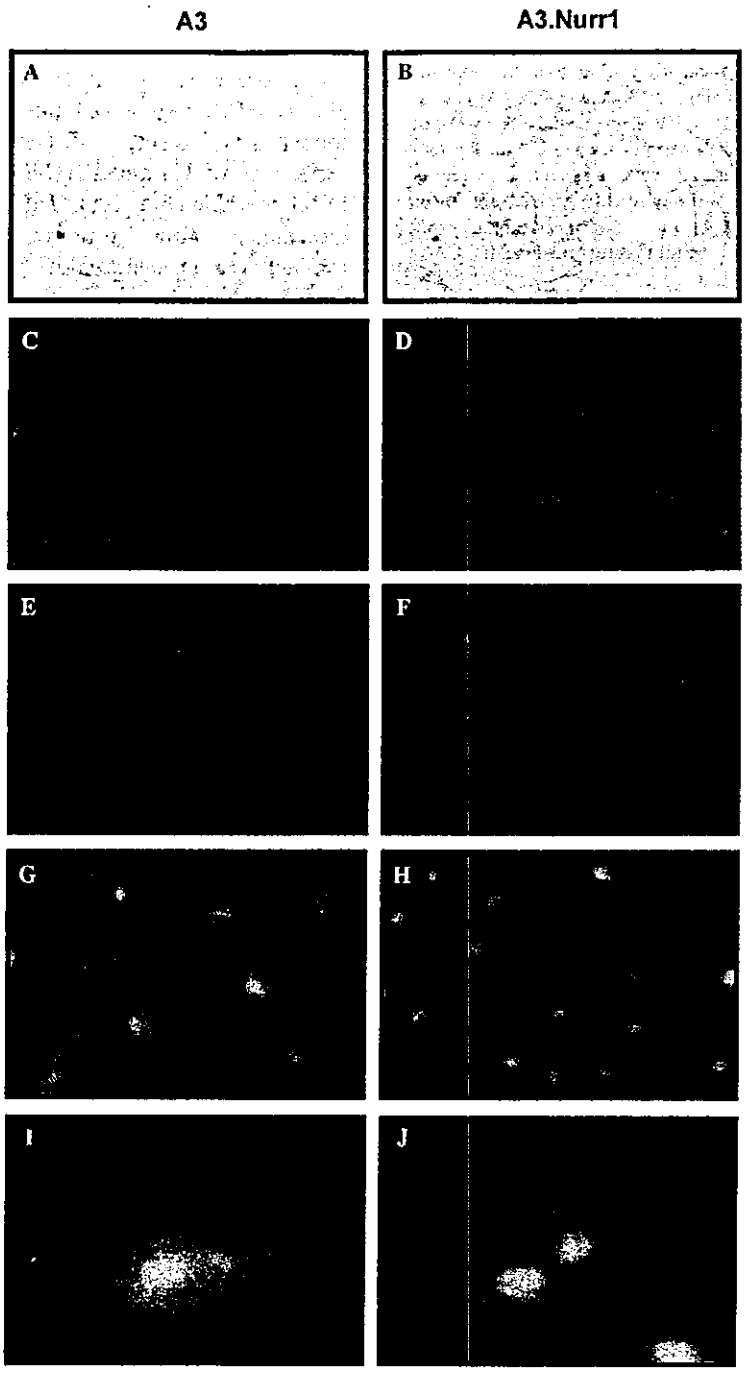

Fig. 1. Nurr1 expression induces morphological differentiation in mouse NSCs. (A,B) Morphological changes are observed in A3 and A3.Nurr1 by phase contrast microscopy. A3 cells showed fibroblast-like shape, bearing occasionally lamellipodia, whereas Nurr1-expressing cells protruded a short pine needle-like protrusion, filopodia. A distinguishable morphology was evident by visualizing the actin cytoskeleton (C,D) and paxillin containing focal adhesion assembly (E,F) in A3 and A3.Nurr1 cells.

overexpressing NSC line. To this end, an amphotropic producer cell line producing LPC-Shh-N retrovirus was selected and expanded. The virus had a titer of 200 CFU/ml for NIH3T3 cells and did not contain helper viruses. Another NSC line C4 was transduced with this retrovirus and selected for stable transfectants with 5 μg/ml puromycin. Colonies were picked after selection for 10 days and tested for the intracellular expression and secretion of Shh-N protein. A clone, C4.Shh, showed intracellular expression of Shh-N proteins (Fig. 4A). On the other hand, endogenous expression of Shh-N proteins could not be detected in parental C4 cells by Western blotting

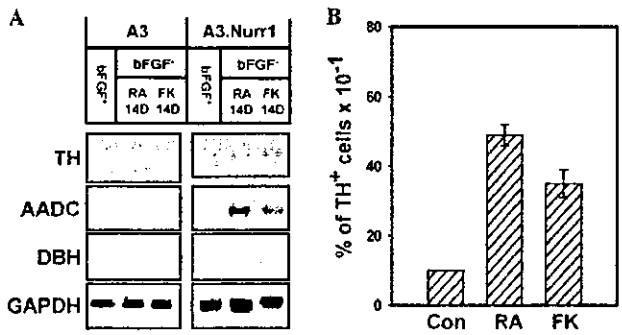

Fig. 2. Effects of retinoic acid and forskolin on Nurr1-dependent TH expression. (A) Expression of genes related to catecholamine-producing neurons in mouse NSCs before and after differentiation. Differentiation was induced by bFGF withdrawal followed by RA or FK treatment and evaluated by semiquantitative RT-PCR. Cells were treated with 0.5 μM RA for 6 days, followed by N2 medium alone for an additional 8 days. In the FK conditions, cells were treated with 5 μM FK for 14 days. The presence of TH transcripts was measured by quantitative RT-PCR analyses and normalized to GAPDH. (B) Histogram demonstrating the percentage of TH^+ cells in cultures of A3.Nurr1 cells in the presence of RA or FK.

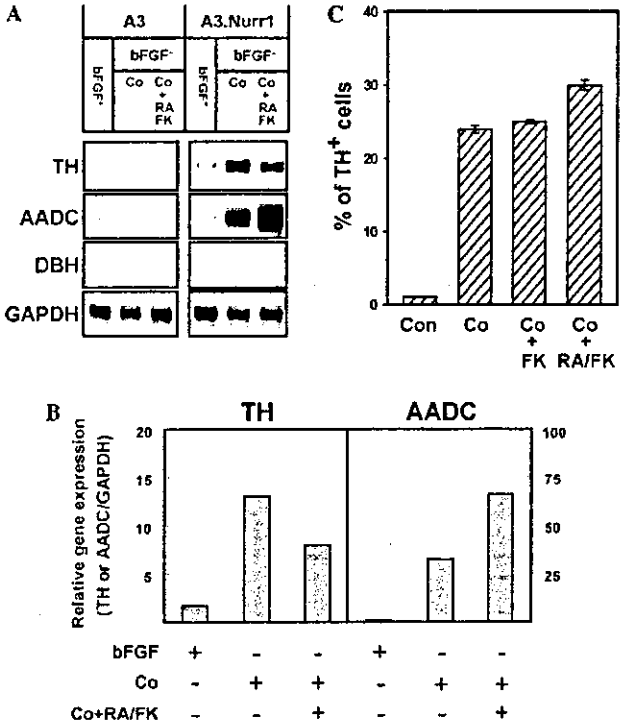

Fig. 3. Nurr1-overexpressing NSCs co-cultured with human fetal astrocytes for TH induction. (A) A3 and A3.Nurr1 cells were co-cultured with human fetal astrocytes and induction of genes related to catecholamine-producing neurons was assessed by RT-PCR. (B) Histogram demonstrating relative expression of TH and AADC mRNA in cultures of A3.Nurr1 cells. The presence of TH transcripts was measured by quantitative RT-PCR analyses and normalized to GAPDH. (C) Nurr1 clones co-cultured with human fetal astrocytes expressed an increase in the number of TH-positive cells. Addition of retinoic acid and/or forskolin slightly enhanced the differentiation of A3.Nurr1 cells into TH-positive cells in co-cultures. The induction of these phenotype was specific to A3.Nurr1 cells and was not observed in parental A3 cells co-cultured with human fetal astrocytes.

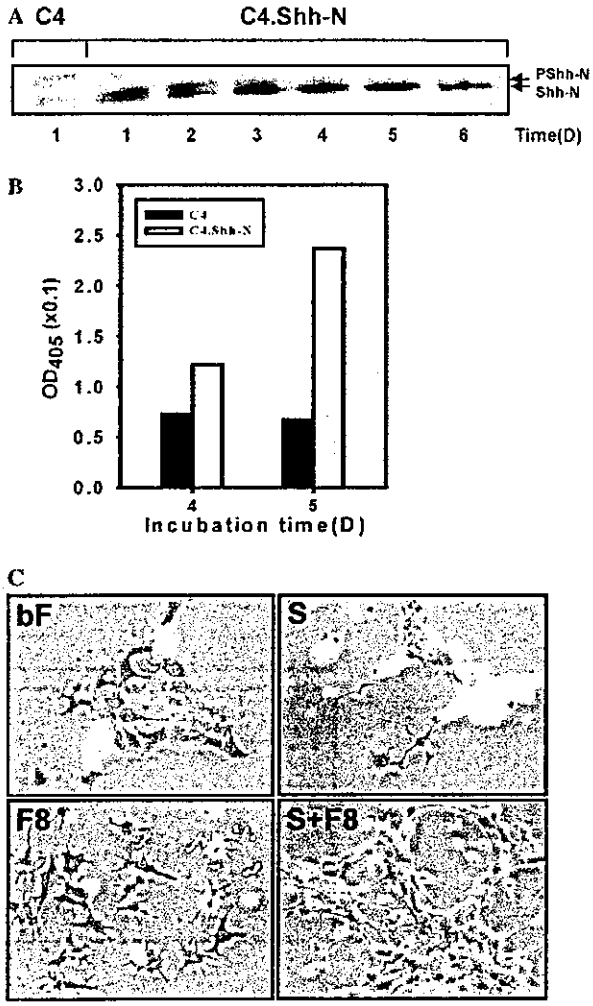

Fig. 4. (A) Western blot analysis with an anti-Shh-N antibody demonstrates intracellular expression of Shh-N proteins (23 kDa) in cell lysates of A3.Nurr1 but not in cell lysates of A3 cells. (B) Secretion of Shh-N proteins. Media conditioned by A3 or A3.Nurr1 cells were assayed for Shh-N proteins by ELISA. (C) Microphotographs showing A3.Nurr1 cells grown in the presence of bFGF, co-culture with C4.Shh cells, or FGF8 (10 ng/ml), and co-culture with C4.Shh cells plus FGF8 (10 ng/ml). Cells maintained a monolayer and extended short neurites around cell periphery when grown in the presence of Shh-N or FGF8 alone for 5 days. However, A3.Nurr1 cells extended many long neurites and arrayed with direction when treated with Shh-N and FGF8 for 5 days. In addition, small clusters of A3.Nurr1 cells were well developed and interconnected by neurites.

(Fig. 4A). ELISA analysis for the medium conditioned by C4.Shh cells demonstrated that Shh-N proteins were secreted by C4.Shh cells (Fig. 4B). In contrast, C4 cells did not secrete a detectable amount of the Shh-N proteins (Fig. 4B).

Shh and FGF8 induces morphological differentiation in NSCs

Mouse NSC line A3 and its Nurr1 clone, A3.Nurr1, were fibroblast-like and polygonal in shape and did not

have long processes (Figs. 1A, B and 4C). Both cells grew rapidly (doubling time of 12 h). However, when NSC lines were grown with FGF8 and insert culture of C4.Shh cells for 3 days, most of the cells stopped dividing, began extending long processes, and developed cell clusters (Fig. 4C). After 5 days, cell clusters increased in size and developed more prominent processes compared with cells grown alone (Fig. 4C). This morphology could be clearly visualized by staining for a neuronal specific marker such as tubulin β III (Figs. 5A and B). Five days after induction of differentiation, almost all the cells displayed a highly mature neuronal morphology, including long, elaborate processes, hypertrophic cell bodies, and developed prominent cell–cell network and cell clusters, and intense levels of tubulin β III immunoreactivity (Figs. 4 and 5). However, A3.Nurr1 cells treated with FGF8 or co-cultured with C4.Shh cells developed long process, hypertrophic cell bodies, but did not form intense network and cell clusters.

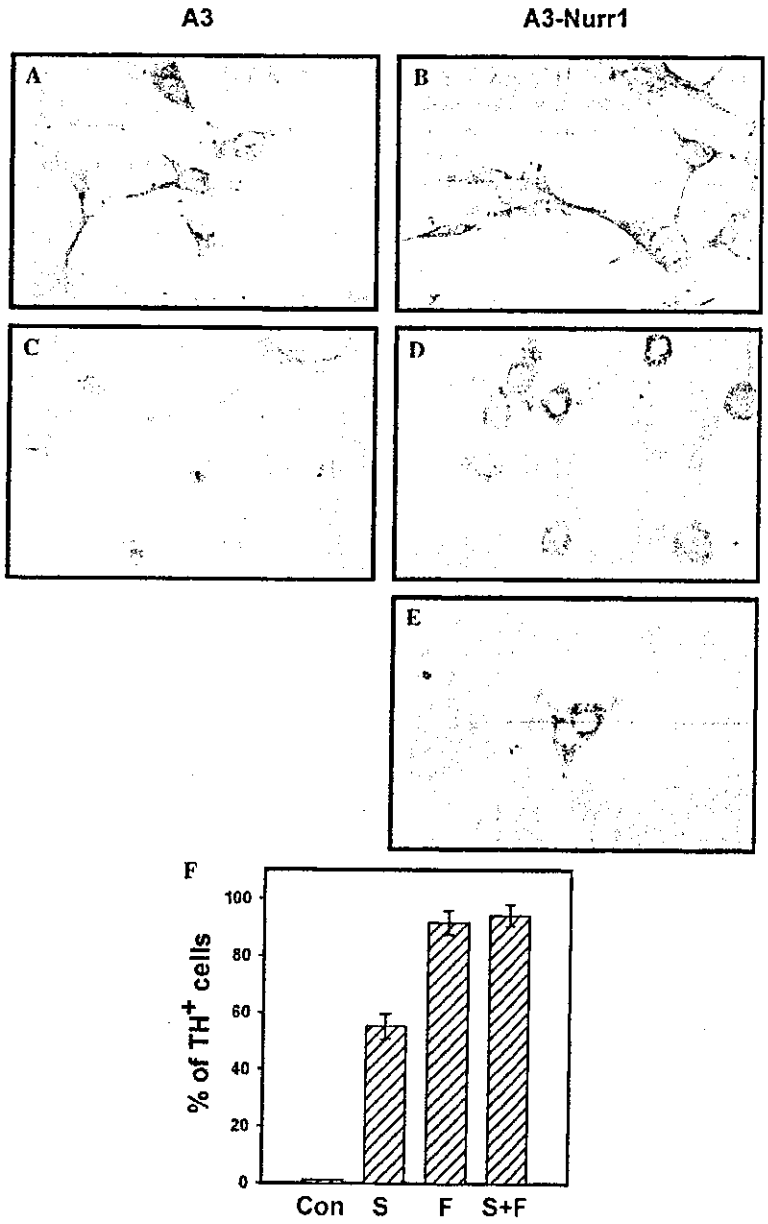

Fig. 5. Immunocytochemical staining for tubulin β III (A,B) and TH (C–E). Treatment with Shh-N and FGF8 induced marked morphological differentiation in both A3 and A3.Nurr1 cells (A,B). However, few TH-labeled cells were observed in parental A3 cells treated with Shh-N and FGF8 for 5 days (C). Compared with parental cells, A3.Nurr1 cells became TH-positive after 5 days when treated with FGF8 alone (D) or with FGF8 (10 ng/ml) plus co-culture of C4.Shh cells (E). (F) Histogram demonstrating the percentage of TH^+ cells in cultures of A3.Nurr1 cells grown alone and grown on a insert culture of C4.Shh cells, FGF8 (10 ng/ml) or FGF8 (10 ng/ml) plus co-culture of C4.Shh cells.

Increase in TH-positive cell numbers by FGF8 and Shh-N

To test whether these changes in morphology were attributed to the differentiation of DA neurons, the cells were examined immunohistochemically. When the cells were grown in the presence of FGF8 and Shh, immunoreactivity of tubulin β III was observed in almost all of the cells in both A3 and A3.Nurr1 cells (Figs. 5A and B). However, immunoreactivity of TH was different in A3 and A3.Nurr1 cells (Figs. 5C–E). The number of TH-positive cells greatly increased with FGF8 and co-culture of C4.Shh-N, as reflected by the increased total number of TH-positive cells ($94.3 \pm 3.7\%$ of total number of cells) in A3.Nurr1 cells. This pronounced effect on the generation of TH-positive cells was specific to Nurr1 overexpressing NSCs (Fig. 5E), but not to parental NSCs (Fig. 5C). Under these conditions, TH-positive cells were occasionally observed but were faintly stained, and the number never exceeded 2% of total cells in A3 cells (Fig. 5C). In addition, while applied as single factor, Shh-N was less effective in inducing DA phenotypes ($55.2 \pm 4.5\%$ of total number of cells) than when added in combination with FGF8, but FGF8 is equally effective ($91.7 \pm 4.2\%$ of total number of cells) when compared to FGF8 and C4.Shh co-cultures (Fig. 5F).

Collectively, the level of TH immunoreactivity increased substantially depending upon the dopaminergic induers used. The percentages of cells stained positive for TH immunoreactivity in the presence of each inducer used were $4.9 \pm 0.3\%$ ($n = 5$) for RA, $3.5 \pm 0.4\%$ ($n = 5$) for FK, $24.7 \pm 2.3\%$ for human fetal astrocyte, and $94.3 \pm 3.7\%$ for co-culture of C4.Shh plus FGF8. These results indicate that Shh-N and FGF8 are specific inducers of DA phenotypes in NSCs.

Discussion

The present study demonstrated that Nurr1 restricts uncommitted multipotent mouse NSCs to neuronal lineage, but it could not induce dopaminergic fate in mouse NSC. The observation that TH gene induction occurs after attainment of a mature neuronal phenotype indicates that Nurr1 alone is not sufficient for activating TH gene expression, but rather requires specific cellular (or neuronal) environments or cofactors. Such cofactors or conditions may be present in Nurr1-overexpressing adult hippocampal precursors [21], embryonic stem cells [23,24], NSC [25,26], mesencephalic cell [17,27], or provided by mesencephalic astrocytes to allow TH induction in mouse neuronal precursors [16]. In addition, it has been demonstrated that Nurr1 can directly activate the TH promoter in a cell line-specific manner [28,29], supporting the view that certain transcriptional conditions are required for TH gene induction by Nurr1.

Various signaling molecules have been extensively tested for their potential to facilitate DA lineage differentiation using ES and progenitor cells [15,30]. We tested if the retinoic acid, forskolin, co-culture with human fetal astrocytes, or the combined treatment with Shh-N and FGF8 can increase induction of TH-positive neurons in the Nurr1-overexpressing NSC clone. For the first time, we tested whether treating A3.Nurr1 cells with combinations of the previously mentioned proliferative, differentiation, and trophic factors could increase the number of TH-positive neurons. Treatments to A3.Nurr1 cells were generally ineffective with bFGF, retinoic acid, and/or forskolin. Thus, these results suggest that bFGF, retinoids, and forskolin do not act as inductive signals in our system. Furthermore, as retinoic acid specifically stimulates RXR receptors [31], which heterodimerize with Nurr1 and/or other nuclear receptors to form transcription-initiating complexes [32], RXR receptor stimulation does not play an important role in enhancing TH-positive neurons, suggesting that TH induction may have acted through distinct mechanism from Nurr1–RXR dimerization pathway.

Previous studies have demonstrated that diffusible and contact-dependent signals from astrocytes [16,17] can increase the number of NSCs differentiating into DA neurons in vitro. In the present study, co-culture via a microporous insert of fetal astrocytes was able to significantly increase the number of TH-positive neurons in A3.Nurr1 cell cultures, suggesting that astrocytes secrete a highly labile diffusible factor that interacts with Nurr1-overexpressing NSC line to generate TH-positive neurons. Collectively, these observations suggested that a factor derived from the primary astrocyte cultures interacted directly or indirectly with Nurr1 to induce TH-expressing neurons. However, because TH expression was limited to a minority subpopulation within a fraction of the Nurr1 clones, some property of the primary cultures or of the Nurr1 clones themselves must have been limiting.

The increase of TH-positive neurons from A3.Nurr1 cells was even more pronounced when Shh-N and FGF8 were applied to the Nurr1-NSC clone during in vitro differentiation. Of signaling molecules treated to NSCs in our system, the combined treatment of Shh-N and FGF8 was the most effective inducer of DA neurons. In this condition, almost all of neuronal clusters contained large numbers of TH-positive neurons and our quantitative analysis showed that approximately $94.3 \pm 0.7\%$ of total A3.Nurr1 cells were TH-positive (Fig. 5). More importantly, induction of TH-positive neurons was evident only in Nurr1 transduced clone, not parental NSCs. These observations therefore do not support the notion that Nurr1 may act downstream of one of these signaling molecules, but rather, suggest that Nurr1 and these molecules may act independently or synergistically to induce the DA lineage differentiation. In addition,

Shh-N and FGF8 simultaneously induced the expression of DA markers and typical neuronal morphology in both NSCs and Nurr1 transduced NSCs, suggesting that these molecules were conductive for both cellular and morphological differentiation of NSCs into DA phenotypes. It will be very interesting in the future to investigate the molecular nature of the signals involved in the generation of DA neurons from NSCs.

In conclusion, our results suggest that Nurr1 induces neuronal differentiation and confers competence to respond to extrinsic signals that induce dopaminergic fate in a mouse NSC line. Moreover, the present study demonstrates that the most potent sources of such signals are Shh-N and FGF8, which induces Nurr1-expressing NSCs to develop into typical DA neurons. Our findings further suggest that Shh-N and FGF8 may be the sources of signals required for the induction of both cellular and morphological differentiation of NSCs into DA phenotypes. Finally, the procedures we describe, taking advantage of the multipotential capacity of NSCs, selector genes such as Nurr1, and in vivo dopaminergic inducers, might be used to engineer neurons with the desired neurochemical phenotypes as a source material for neuronal transplantation in the treatment of neurodegenerative diseases. Taken together, these observations suggest that our immortalized NSCs can serve as an excellent model for understanding mechanisms that regulate specification of ventral midbrain DA neurons and as an unlimited source of DA progenitors for treating Parkinson's disease patients by cell replacement.

Acknowledgments

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GENE THERAPY FOR PARKINSON'S DISEASE USING AAV VECTORS

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1. INTRODUCTION

Parkinson's disease (PD) is a common disorder characterized by progressive disturbance in the control of movement, and the symptoms result from a progressive loss of dopaminergic neurons in the substantia nigra. These neurons send dopamine down to their nerve endings that are located in the striatum. The severity of PD is proportional to the decline in the amount of dopamine in the striatum. Dopamine is produced from tyrosine, which is first hydroxylated and converted to L-dopa by tyrosine hydroxylase (TH) in the presence of tetrahydrobiopterin (BH_4) as a cofactor. L-dopa is subsequently decarboxylated into dopamine by a second enzyme called aromatic L-amino acid decarboxylase (AADC). The BH_4 is synthesized from guanosine triphosphate (GTP) in the TH-containing dopamine neurons where GTP cyclohydrolase I (GCH) catalyzes the first and rate limiting step of BH_4 biosynthesis. The GCH is considered to regulate the TH activity via regulation of BH_4 biosynthesis, and thus controls indirectly dopamine production.

Patients with PD can be treated with oral administration of L-dopa, but unfortunately, this therapy usually becomes less effective with progression of the disease and must often be discontinued due to the numerous serious side effects. Local production of dopamine in the striatum can be obtained by grafting of fetal mesencephalic neurons into the patient's brain, but serious practical and ethical issues are associated with this approach. Gene therapy offers a novel potential alternative to the treatment of PD. Initial gene therapy strategies for PD have focused on modifying cells ex vivo by delivering the gene for TH to these cells. However, a potentially simpler and more practical gene therapy approach involves delivering the gene directly into the brain. A promising approach for achieving this is the use of a gene delivery vehicle based on adeno-associated virus (AAV), a common virus that has never been associated with human disease.

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2. GENE TRANSFER INTO NEURONS USING AAV VECTORS

AAV vectors and lentivirus vectors can transduce non-dividing neurons and long-term gene expression can be obtained. As for lentivirus vectors, human immunodeficiency virus (HIV) vectors are currently well investigated. However, the safety issue of HIV vectors is serious problem when chronic diseases are target. On the other hand, AAV vectors, derived from non-pathogenic virus, possess several unique properties and are potentially useful gene transfer vehicles for gene therapy. Therefore, AAV vectors are currently most appropriate for gene therapy of neurological diseases.

The AAV genome consists of 4681 nucleotides (nt) of single-stranded DNA, including two inverted terminal repeats (ITRs) of 145 nt long, which serve as an origin of replication and are also essential for packaging and rescue. The advantages of AAV vectors include lack of any associated disease with a wild-type virus, broad host cell range, integration of the gene into the host genome, and the ability to transduce non-dividing cells. AAV vectors are suitable for in situ transduction, and therefore, neurons, muscles, and hepatocytes are considered to be appropriate target cells. Most part of the transferred genes with AAV vectors are initially present as an episomal form, and the transgenes are expressed after the conversion to double-stranded DNA. Transgenes are believed to integrate gradually into the host cell genome, leading to their stable expression. Since the AAV vectors do not contain viral genes, immune response should not occur against the transduced cells. It is also important that cotransduction with multiple AAV vectors is efficient, because the capacity of gene insertion is limited for small-sized AAV vectors (approximately, up to 4.5 kb of a fragment including promoter and polyadenylation signal sequences can be inserted between the ITRs).

When primary cultured rat striatal cells were transduced with AAV vectors containing LacZ gene (AAV-LacZ), roughly 30% of striatal cells were positive for ß-galactosidase activity, and about one week was required for maximum expression[1]. The AAV-LacZ vector was also injected into the striatum of normal adult rats. When the rats were sacrificed at 1 week postinjection, LacZ-positive cells were clearly identified at the injection site within the striatum without any background staining using X-gal histochemistry.

3. DOPAMINE-SUPPLEMENT GENE THERAPY USING AAV VECTORS IN PARKINSONIAN RATS

To prepare a rodent model of PD, neurotoxic 6-hydroxydopamine (6-OHDA) was stereotaxically injected into the left medial forebrain bundle (MFB) to destroy the nigro-striatal pathway of dopaminergic neurons unilaterally. This MFB lesion causes the decrease of dopamine in the striatum like PD. The dopamine receptors of striatal cells in the lesioned side become supersensitive to dopamine. After the intraperitoneal administration of dopamine agonist, apomorphine, unbalance of motor function is induced, and the rats show rotational behavior. The dopamine content in the striatum can be estimated by counting the rotation after apomorphine administration. As a model experiment of gene therapy, AAV vectors were stereotaxically injected into the

denervated striatum of 6-OHDA-lesioned rats. We showed that the coexpression of TH and AADC using two AAV vectors resulted in more effective dopamine production and more remarkable behavioral recovery in 6-OHDA-lesioned parkinsonian rats, than the expression of TH alone[1]. Not only TH and AADC but also BH_4 and GCH levels are reduced in parkinsonian striatum. Therefore, we next investigated whether transduction with separate AAV vectors expressing TH, AADC, and GCH was far more effective for gene therapy of PD than the dual transduction. First, in vitro experiments showed that the triple transduction with AAV-TH, AAV-AADC, and AAV-GCH resulted in greater dopamine production than the double transduction with AAV-TH and AAV-AADC in 293 cells. In parkinsonian rats treated with the triple transduction, dual immunofluorescence study showed that the striatal TH-positive cells were also positive for MAP2 (a neuronal marker), but not for GFAP (a glial marker), indicating that the transduced cells were neurons. In addition, efficient co-expression was confirmed by a double-labeling with both anti-TH and anti-AADC antibodies, or with both anti-TH and anti-GCH antibodies, which showed that more than 90% TH-IR cells were also positive for AADC and GCH. Furthermore, the triple transduction enhanced the BH_4 and dopamine production in denervated striatum of parkinsonian rats and improved the rotational behavior of the rats more efficiently than did the double transduction[2]. Behavioral recovery persisted for at least 18 months after stereotaxic intrastriatal injection. These results suggest that GCH as well as TH and AADC is important for effective gene therapy of PD. This finding would be valuable for developing more efficacious gene therapy strategies for PD. It is worthy of note that multiple genes can be efficiently delivered and expressed in neurons in vivo using separate AAV vectors as expected.

4. BEHAVIORAL RECOVERY IN A PRIMATE MODEL OF PARKINSON'S DISEASE BY GENE THERAPY WITH AAV VECTORS EXPRESSING DOPAMINE-SYNTHESIZING ENZYMES

To examine whether the above findings observed in parkinsonian rats can be extrapolated to humans, primate model experiments are very important as a preclinical study. The most critical difference between rats and monkeys is the size of striatum. Cynomolgus macaques (Macaca fascicularis) were chronically treated with 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) to make bilateral striatal lesions. After animals became behaviorally stable for two months, mixture of three separate AAV vectors expressing TH, AADC, and GCH, respectively, were injected into the unilateral putamen stereotaxically. Coexpression of TH, AADC, and GCH in the unilateral putamen resulted in remarkable improvement in manual dexterity on the contralateral to the AAV-TH/-AADC/-GCH-injected side (S. Muramatsu, et al.: submitted). Fine motor task consisting of capturing four raisins improved on the contralateral hand. Systemic administration of apomorphine induced turning of body toward the ipsilateral side, suggesting that supersensitivity in the AAV-vector-injected putamen was reduced. TH-immunoreactive (TH-IR), AADC-IR, and GCH-IR cells were present in a large region of the putamen. Microdialysis demonstrated that concentrations of DA and its metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and 3 methoxytyramine, in the AAV-TH/-AADC/-GCH-injected putamen were remarkably higher compared with the control side. No adverse behavioral or histopathological effects were observed. Our results show that AAV vectors efficiently introduce DA-synthesizing enzyme genes into the striatum of

primates with restoration of motor functions. This triple transduction strategy is a feasible and novel treatment of PD.

5. FUTURE PERSPECTIVES

There are two major approaches to the treatment of PD; i.e. gene therapy and cell therapy. Cell-mediated gene therapy is a combination of both approaches, and is based on the amplification and genetic modification of cells in vitro prior to transplantation to the brain. Therapeutic genes can be introduced into the brain either by direct in vivo injection of vectors, or by ex vivo transduction.

Although gene therapy may be effective to improve the clinical manifestations of PD, the treatments aimed at augmenting striatal dopamine production do not halt the progressive degeneration of nigral dopaminergic neurons. Therefore, another approach seems to be important to obtain the long-term therapeutic effects of gene therapy for PD. The glial cell line-derived neurotrophic factor (GDNF), a potent neurotrophic factor for dopaminergic neurons, is a promising therapeutic molecule for limiting the neuronal damage caused by PD[3]. The combination of dopamine-supplement gene therapy and GDNF gene therapy would be a logical approach for the treatment of PD.

As for cell therapy, besides embryonic mesencephalic cells, other sources of catecholamine producing cells, such as adrenal medulla, sympathetic ganglia, and carotid body, have also been considered potentially useful for cell replacement therapy of PD. Neural stem cells derived from the cerebellum of the mouse can be induced to develop into dopaminergic neurons by the overexpression of the nuclear receptor Nurr1 and factors derived from local type 1 astrocytes. Neural progenitors may be applied to cell-mediated therapy, since they are brain-derived and can be expanded and genetically modified in vitro. When transplanted to the brain, neural progenitors undergo glial and neuronal differentiation depending on local environment. In the future, dopaminergic neurons will be generated from human embryonic (pluripotent) stem cells for clinical use in cell replacement therapy of PD. Genetic manipulation will promote the conversion of human stem cells to dopaminergic neurons, and further augment the ability of dopamine production with an appropriate regulatory system. Several approaches should be explored toward the realization of novel and efficient therapy of PD.

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Adeno-associated virus vectors for gene transfer to the brain

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Abstract

Gene therapy is a novel method under investigation for the treatment of neurological disorders. Considerable interest has focused on the possibility of using viral vectors to deliver genes to the central nervous system. Adeno-associated virus (AAV) is a potentially useful gene transfer vehicle for neurologic gene therapies. The advantages of AAV vector include the lack of any associated disease with a wild-type virus, the ability to transduce nondividing cells, the possible integration of the gene into the host genome, and the long-term expression of transgenes. The development of novel therapeutic strategies for neurological disorder by using AAV vector has an increasing impact on gene therapy research. This article describes methods that can be used to generate rodent and non-human primate models for testing treatment strategies linked to pathophysiological events in the ischemic brain and neurodegenerative disorders such as Parkinson's disease.

1. Introduction

The adeno-associated virus (AAV)-based vector system has gained attention as a potentially useful alternative to the more commonly used retroviral and adenoviral vectors for human gene therapy. AAV is a dependent parvovirus with a single-stranded linear genome that contains two open reading frames (*rep* and *cap*) [1]. The genome encodes four replication proteins (Rep78, Rep68, Rep52, and Rep40) and three capsid proteins (Cap: VP1, VP2, and VP3). Recombinant AAV (rAAV) is a promising gene delivery vehicle for gene therapy. Their cloning capacity of 4.5 kb can accommodate a variety of cDNAs. Furthermore, no diseases associated with AAV have been reported in either human or animal populations. The properties that make AAV an ideal vector for gene therapy include its ability to infect both dividing and nondividing cells and the longevity of expression in tissues such as brain, skeletal muscle, and liver.

The preparation of AAV vector for gene therapy study of neuronal diseases is greatly facilitated. Five primate AAV serotypes have been characterized in the literature and are designated as AAV types 1–5 (AAV1–5). There is divergence in homology and tropism for various AAV serotypes [2–5] (Table 1). Recent findings have shown that recombinant AAV5 (rAAV5) can transduce both neuronal cells and glial cells in a highly efficient manner, while rAAV2 preferentially transduces neuronal cells. Cerebral parenchymal gene transduction of rAAV5 compared with rAAV2 was greatly enhanced with the diffuse and widespread pattern of transduction [6]. Because the homology with both viral inverted terminal repeats and capsid protein is only about 60% between AAV2 and AAV5 [7], it is understood that the capsid structure could be responsible for the improved transduction efficiency of AAV5 [8]. Here we show methods of gene transfer to the brain for investigating gene therapies for neuronal disorders.

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Table 1
Characteristics of primate AAV serotypes

Serotype	Host	Homology with AAV-2 Cap	Heparin affinity	Receptor	Major tissue tropism
1	Monkey	High (83%)	–	(Unknown)	Skeletal muscle
2	Human	(100%)	+	Heparin sulfate	Neuron
3	Human	High (87%)	±	(Unknown)	Neuron
4	Monkey	Low (62%)	–	Sialic acid (α2–3)	Ependymal cell
5	Human	Low (58%)	–	Sialic acid (α2–3, α2–6)	Neuron, astrocyte, retina, airway, liver

2. AAV vector preparation

2.1. Principle of triple plasmid transfection system

The minimum sets of regions in helper adenovirus that mediate AAV vector replication are the E1, E2A, E4, and VA [9]. A human embryonic kidney cell line 293 encodes the E1 region of the Ad5 genome [10]. The helper plasmid assembling E2A, E4, and VA regions (Ad-helper plasmid) is cotransfected into the 293 cells, along with plasmids encoding the AAV vector genome (vector plasmid) and *rep* and *cap* genes (AAV-helper plasmid). AAV vector is produced as efficiently as when adenovirus infection is employed as a helper virus. Elimination of the heat inactivation for the contaminated adenovirus can improve yield of the recombinant AAV. Furthermore, contamination of most adenovirus proteins can be avoided in AAV vector stock made by this helper virus-free method.

One of the limitations of rAAV vector systems for gene therapy applications has been the difficulty in producing the vector in sufficient quantity for adequate evaluation. Because the AAV Rep proteins are cytotoxic, it is not easy to establish stable cell lines that express them constitutively. The conventional method for generating vector stocks consists of cotransfection with a plasmid containing the vector genome and a *trans*-acting helper construct for *rep* and *cap* expression. Recent improvements in vector production include the development of packaging cell lines expressing Rep and Cap [11–14] and ways for inducible expression and regulation of Rep and Cap [15–18]. However, establishing stable cell lines is still difficult because the early expression of Rep proteins is toxic to cells [19].

2.2. Reagents

Reagents used were as follows: Ad-helper plasmid DNA (pAdeno); AAV-helper plasmid DNA (pHLP for rAAV2, pAAV5RepCap for rAAV5); vector plasmid harboring gene of interest flanked with inverted terminal repeats (ITRs); 290 mM NaCl, 50 mM Hepes buffer, 1.5 mM Na_2HPO_4, pH 7.10 (2× HBS); 300 mM $CaCl_2$; 293HEK cells; Dulbecco's modified Eagle's medium (DMEM/F12) culture medium, 10% fetal calf serum (FCS); phosphate-buffered saline (PBS); 1 M Hepes buffer, pH 7.4; 100 mM Tris–HCl, pH 8.0, 150 mM NaCl (TBS); 0.5 M EDTA, pH 8.0; 1 M $MgCl_2$; benzonase nuclease; 50 mM Hepes, pH 7.4, 0.15 M NaCl, 25 mM EDTA (HNE); 10% deoxycholate in water (note: HNE is not applicable); CsCl in HNE (1.25 g/ml); and CsCl in HNE (1.50 g/ml).

2.3. Plasmids

The AAV vector plasmid harbors a transgene expression cassette flanked by ITRs. AAV2-helper plasmid pHLP harboring Rep and Cap was previously reported as pHLP19 [20]. AAV5-helper plasmid pAAV5RepCap is identical to 5RepCapB [7]. Ad-helper plasmid pAdeno, identical to pVAE2AE4-5, encodes the entire E2A and E4 regions and VA RNA I and II genes [9].

Table 2
Large-scale triple plasmid transaction into 293 cells for the production of recombinant AAV

Day	Procedure	Material	Scale and volume	
			225-cm^2 flask	6320-cm^2 flask[a]
0	Plating	DMEM/F12 medium, 10% FCS	40 ml	1120 ml
1–2	Medium exchange (1 h before transfection)	DMEM/F 12 medium, 10% FCS	20 ml	560 ml
	Transfection	Plasmid DNA (vector, pHLP, pAdeno)	23 μg each	644 μg each
		0.3 M $CaCl_2$	4 ml	112 ml
		2× HBS	4 ml	112 ml
	Medium exchange (4–6 h after incubation)	DMEM/F12 medium, 2% FCS	40 ml	1120 ml
4–5	Harvest	0.5 M EDTA	1 ml	28 ml

[a] The 10-tray Nunc Cell Factory system is equivalent to 28 225-cm^2 flasks.

2.4. Transfection and viral extraction

This protocol is for the transfection of one 225-cm^2 flask. For additional flasks or different volumes of capacity, volumes can be increased on a linear basis (Table 2). The process is linearly scaleable to a 10-tray flask (Nunc Cell Factories 164327; Nalge Nunc, Naperville, IL).

Proceed as follows. Trypsinize 293 cells and plate 5×10^6 cells per 225-cm^2 flask to achieve a monolayer at 20–40% of confluency when cells initially attach to the surface of the flask. The volume of medium per flask is 40 ml. Try to avoid plating clumps of cells and make sure that cells are distributed evenly across the plate. Even cell density in all areas of the plate is essential for high yield and can be attained by moving the plates of newly plated cells gently in a cross pattern before the cells attach. Place plates in a 5% CO_2 incubator and let the cells grow to a confluency of 60–70% over the next 24–48 h. At 1 h before the transfection, exchange half of the medium in tissue culture flasks with fresh DMEM/F12 culture medium containing 10% FCS. Add 23 μg each of vector and helper plasmids to 4 ml of 300 mM $CaCl_2$. Gently add this solution to 4 ml of 2× HBS and immediately mix by gentle inversion three times. Immediately pipette this mixture into a 225-cm^2 flask of 293 cells containing 40 ml of DMEM/F12 culture medium plus 10% FCS and swirl to produce a homogeneous solution. Immediately transfer plates to a 5% CO_2 incubator and incubate at 37 °C for 4–6 h. Do not disturb plates during this period. At the end of the incubation, replace medium with prewarmed DMEM/F12 culture medium containing 2% FCS. Three days after the transfection, add 1 ml of 0.5 M EDTA to each flask and incubate for 3 min at room temperature. Collect cell suspension and centrifuge at 300*g* for 10 min. Remove supernatant and resuspend cell pellet in 2 ml of TBS. Freeze and thaw cells suspended in TBS three times by placing them alternately in a dry ice/ethanol bath until completely frozen and in a 37 °C-water bath until completely thawed. Immediately transfer sample back to ice bath when completely thawed. Remove tissue debris by centrifuging at 10,000*g* for 10 min and collect supernatant.

2.5. AAV vector purification

For the material obtained from 28 flasks (225 cm^2) or a 10-tray flask (6320 cm^2), add 5 mM $MgCl_2$ (final concentration) with 5000 units of benzonase nuclease and incubate for 30 min at 37 °C. Then add 5 mM EDTA (final concentration) and incubate with 0.5% (final concentration) deoxycholic acid (Sigma D6750; Sigma, St. Louis, MO) for 30 min at 37 °C. Remove debris by centrifuging at 10,000*g* for 2 min followed by filtration with low-protein-binding 5-μm syringe filter (Sterile Acrodisc Syringe Filter; Pall Gelman Laboratory, Ann Arbor, MI). Load the filtrated crude lysate onto a two-tier CsCl gradient (1.25 and 1.50 g/ml) in HNE buffer. Spin the gradient at 25,000 rpm for 3 h at 16 °C in a SW 28 rotor (Beckman Instruments, Palo Alto, CA). Collect the virus-rich fraction while measuring the refractive index (RI; 1.371–1.380) and mix the putative virus fraction with CsCl solution (1.25 g/ml) in HNE buffer to load onto a CsCl solution (1.50 g/ml) in HNE buffer. Spin the gradient at 65,000 rpm for 3 h at 16 °C in a VTi 65.2 rotor (Beckman Instruments). Collect 0.5 ml each of the fractions from the bottom of the tubes and select the virus-rich fraction by measuring RI, by semiquantitative PCR analysis, or by quantitative DNA dot-blot hybridization. By using dialysis cassette Slide-A-Lyzer (Pierce, Rockford, IL), desalt the virus-rich fraction by three cycles of dilution with 300 ml of HNE buffer. Concentrate the material to 50 μl with Ultrafree-4 (Millipore, Bedford, MA) according to the manufacturer's instruction. Yield of the virus is transgene and construct dependent. The final titer of rAAV2 is ca. 1×10^{13} particles from 28 flasks (225 cm^2) or a 10-tray flask as determined by quantitative DNA dot-blot hybridization or Southern blotting. The yield of rAAV5 is usually ca. 1×10^{14} particles, which is higher than that of rAAV2 in the same scale of transfection. In the case of AAV2 vector expressing *lacZ*, vector production can be functionally assessed by titration with 293 cells. For AAV5, the C2C12 myoblasts (CRL-1772; ATCC, Monassus, VA) can be used for the assay because of their enhanced transduction in differentiated myocytes [8]. Differentiation of the cells is induced by culturing the cells in 10% horse serum. Note that the C2C12 myoblasts should be cultured in confluency of less than 50%. The transduced cultures are incubated for 24 h at 37 °C before fixation and X-Gal staining. The stained cells are counted under light microscopy. The genome particle to functional ratio is determined as the level of total genomes divided by that of function units (X-Gal staining) to verify the infectivity.

Many variations of the AAV propagation protocols that we have used could be applied. As an alternative to conventional CsCl gradient centrifugation, methods with iodixanol and a heparin column affinity purification [21], single-step column purification [22], or high-performance liquid chromatography (HPLC) [23] have been reported.

3. Gene transfer to gerbil hippocampus for the treatment of reperfusion injury

3.1. Principle

Ischemic neuronal disease remains a major brain disorder that often renders patients severely impaired and permanently disabled. Despite multiple clinical