# Neuroprotection and Neuronal Differentiation Studies Using Substantia Nigra Dopaminergic Cells Derived from Transgenic Mouse Embryos

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The major pathological lesion of Parkinson's disease (PD) is the selective cell death of dopaminergic (DA) neurons in substantia nigra (SN). Although the initial cause and subsequent molecular signaling mechanisms leading to DA cell death underlying the PD process remain elusive, brain-derived neurotrophic factor (BDNF) is thought to exert neuroprotective as well as neurotrophic roles for the survival and differentiation of DA neurons in SN. Addressing molecular mechanisms of BDNF action in both primary embryonic mesencephalic cultures and *in vivo* animal models has been technically difficult because DA neurons in SN are relatively rare and present with many heterogeneous cell populations in midbrain. We have developed and characterized a DA neuronal cell line of embryonic SN origin that is more accessible to molecular analysis and can be used as an *in vitro* model system for studying SN DA neurons. A clonal SN DA neuronal progenitor cell line SN4741, arrested at an early DA developmental stage, was established from transgenic mouse embryos containing the targeted expression of the thermolabile SV40Tag in SN DA neurons. The phenotypic and morphological differentiation of the SN4741 cells could be manipulated by environmental cues *in vitro*. Exogenous BDNF treatment produced significant neuroprotection against 1-methyl-4-phenylpyridinium, glutamate, and nitric oxide-induced neurotoxicity in the SN4741 cells. Simultaneous phosphorylation of receptor tyrosine kinase B accompanied the neuroprotection. This SN DA neuronal cell line provides a unique model system to circumvent the limitations associated with primary mesencephalic cultures for the elucidation of molecular mechanisms of BDNF action on DA neurons of the SN.

*Key words: neuroprotection; BDNF; substantia nigra; dopaminergic neuron; Parkinson's disease; transgenic mice; neuronal differentiation; conditional immortalization*

The neuropathological symptoms of Parkinson's disease (PD) result from the greater than normal selective degeneration of substantia nigra (SN) dopaminergic (DA) neurons during aging. In PD the initial cause or causes and molecular mechanisms leading to the DA cell death are unknown. Treatment to prevent DA cell loss in PD is not available. Because of the paucity of DA neurons and the presence of numerous other cell populations in SN, it has not been possible to definitively address the molecular mechanisms of both DA neuronal survival and differentiation. Thus, it is crucial to acquire an abundant source of homogeneous DA neurons *in vitro* for the molecular dissection of SN DA neurons. To generate a consistent and abundant source of SN DA neurons *in vitro*, several approaches, using developmental, anatomical, somatic, or genetic manipulations, have been investigated. First, Hynes et al. (1995) tried to recapitulate the ontogeny of SN DA neuronal differentiation in explant cultures. Sonic hedgehog can induce effectively DA neurons in rat embryonic forebrain/midbrain explant. But, to practically manipulate the DA neuronal differentiation *in vitro*, it is essential to elucidate the true identity of the DA progenitor cells present in the brain explants and establish them as a cell culture. The second approach was the purification of fluorescence-labeled DA neurons from embryos by flow cytometry (Kerr et al., 1994). However, both the contamination with non-DA neurons and the low yield requiring a large number of embryos made this approach impractical for a routine pure DA neuronal culture. The third approach was done by somatic cell fusion of embryonic mesencephalic culture with the murine tumor cell line N18TG2 (Choi et al., 1991; Crawford et al., 1992). Although both the hybrid cell lines maintained some limited DA properties, the major drawback of these hybrid cell lines was either the presence of noradrenergic nature caused by the expression of dopamine-$\beta$-hydroxylase or the obscure genetic makeup derived from the parental neuroblastoma genome. In the final approach, the midbrain progenitor cells were randomly immortalized by introducing an oncogene via retroviral infection (Anton et al., 1994). Apparently the lack of a cell type specificity in the retroviral vector made it extremely difficult to immortalize specifically DA progenitor cells in the heterogeneous mesencephalic cultures. Therefore, this paper describes a unique SN-derived DA progenitor cell line developed via combined genetic and developmental manipulations by using a DA neuron-specific promoter (Min et al., 1994), the temperature-sensitive mutant form of an oncogene (Jat and Sharp, 1989), and the anatomical dissection of transgenic embryonic SN.

The development of this cell line made it possible to address the issues of neuroprotection in SN DA neurons. In human, nonhuman primate, and mouse the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) produces biochemical

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and neuropathological changes very similar to those observed in idiopathic PD (Burns et al., 1983; Gerlach and Riederer 1996). In the *in vitro* model of PD using primary mesencephalic culture, 1-methyl-4-phenylpyridinium ($MPP^+$), the active metabolite of MPTP, is selectively taken up by DA neurons and results in the selective neurotoxicity (Nicklas et al., 1985; Krueger et al., 1990). Thus, a close molecular examination of the MPTP model of PD may provide important new insights into the molecular pathogenesis of the DA cell death in PD. Recent studies show that one member of the neurotrophin family, brain-derived neurotrophic factor (BDNF), exerted neuroprotection against $MPP^+$-induced neurotoxicity in primary mesencephalic cultures and animal models of PD (Hyman et al., 1991; Knusel et al., 1991; Altar et al., 1994; Yoshimoto et al., 1995). However, the definitive molecular mechanisms for neuroprotection have yet to be firmly established using an appropriate model system. Therefore, we have characterized and tested the SN-derived DA neuronal cell line as a model for the investigation of the BDNF-regulated neuroprotection at the molecular level.

## MATERIALS AND METHODS

*Generation of transgenic mice carrying TH-SV40Tag-tsA58 fusion gene.* To establish transgenic mice carrying a DNA construct consisting of 9.0 kb of 5′ TH promoter region fused to the temperature-sensitive mutant form of an oncogene, SV40Tag-tsA58, we first constructed the *TH9.0-SV40Tag-tsA58* (Fig. 1*A*). A 2.7 kb *Bgl*I/*Bam*HI early region coding sequence (from pMFSV-tsA58-T; kindly obtained from Dr. H. Federoff, University of Rochester, Rochester, NY) spanning the SV40Tag-tsA58 was cloned into the *Eco*RV site of pTH9000 (Min et al., 1994) containing a 9.0 kb TH promoter region using blunt-ended ligation. The resulting 11.7 kb of fusion gene was isolated from the plasmid vector by restriction digestion with *Hin*dIII. Transgenic mice were generated as described before (Min et al., 1994; Son et al., 1996b). Briefly, the 11.7 kb insert was purified by 0.8% agarose gel electrophoresis followed by electroelution, and subjected to cesium chloride gradient centrifugation as previously described. The purified DNA was microinjected at a concentration of 2 ng/μl into the pronuclei of (CBA/J × C57BL/6J)F2 mouse zygotes. The established two transgenic founders, TA58-#8 and TA58-#13 carrying the TH9.0-SV40Tag-tsA58 fusion gene, were maintained by continuous breeding with F1 hybrid (CBA/J × C57BL/6J). To identify transgenic progeny, genomic DNA isolated from tail tissue was used for Southern blot (*Bam*HI digestion) using TH 9.0 kb as a probe or PCR analysis (primers: nucleotides −493 ~ −473 and −171 ~ −151).

*Establishment of SN DA cell line SN4741.* For the establishment of DA progenitor cell lines, embryonic day 13.5 (E13.5) transgenic embryos from TA58-#8 were obtained. Mesencephalic SN regions from E13.5 embryos were surgically removed under sterile condition in Leibowitz's L-15 media, using the *Atlas of the Prenatal Mouse Brain* (Schambra et al., 1992) as a guide. Because 50% of the embryos were transgenic, the whole litter was used for each dissection without selection of transgenic littermates. Only DA cells from the transgenic embryos would be immortalized and survived in successive cultivation steps. For the isolation of DA cells of SN–ventral tegmental area (VTA) complex from the E13.5 mesencephalon, the transgenic embryos were cut vertically below the base of the brain just above the pigmented eye with extra caution to prevent damage to the ventral mesencephalon. The skin and meninges were carefully removed from the embryonic brain. The ventral mesencephalon, above the mesencephalic flexure, was first dissected by two cuts through both the dorsal surface of the tectum and the thalamus. Then, from the ventral portion, the butterfly-shaped SN-VTA region was removed using surgical knives. The VTA was separated from the residual SN by the removal of the middle part of the butterfly shaped SN-VTA region. SN tissues were cut into small pieces, mechanically triturated in Leibowitz's L-15 (Life Technologies, Gaithersburg, MD) containing Trypsin-EDTA (final concentration, 0.1%), incubated at 37°C for 30 min, and the reaction stopped by adding RF medium (DMEM supplemented with 10% fetal calf serum, 1% glucose, penicillin-streptomycin, and L-glutamine). The cells were pelleted and cultured at 33°C with 5% $CO_2$ in RF medium at least 3–5 weeks. Media was replaced every 4 d. The dispersed primary neuronal cells from the transgenic embryos were grown through repeated passages for 3–4 months to establish a pure cell population. For cloning purposes, cloning cylinders (Bel-Art Products, Pequannock, NJ) were used. The cloning procedure was performed at least three times by plating on 24-well plates coated with polyornithine (Sigma, St. Louis, MO) (1 mg/ml). The clonal origin of a colony was monitored and confirmed by microscopic observation during its colony formation and reconfirmed by TH and SV40Tag immunostaining using slide cultures (Lab-Tek chamber slide; Nunc, Naperville, IL). Locus coeruleus-derived TH-positive cell line was also developed in a similar way from the same transgenic embryos.

*SN DA cell line culture and coculture conditions.* The SN DA neuronal cell line was cultured at 33°C with 5% $CO_2$ in RF medium containing D-MEM supplemented with 10% FCS (Irvine Scientific, Santa Ana, CA), 1% glucose, penicillin-streptomycin, and L-glutamine. The medium was replaced every 4 d. When Lab-Tek chamber slides were used, they

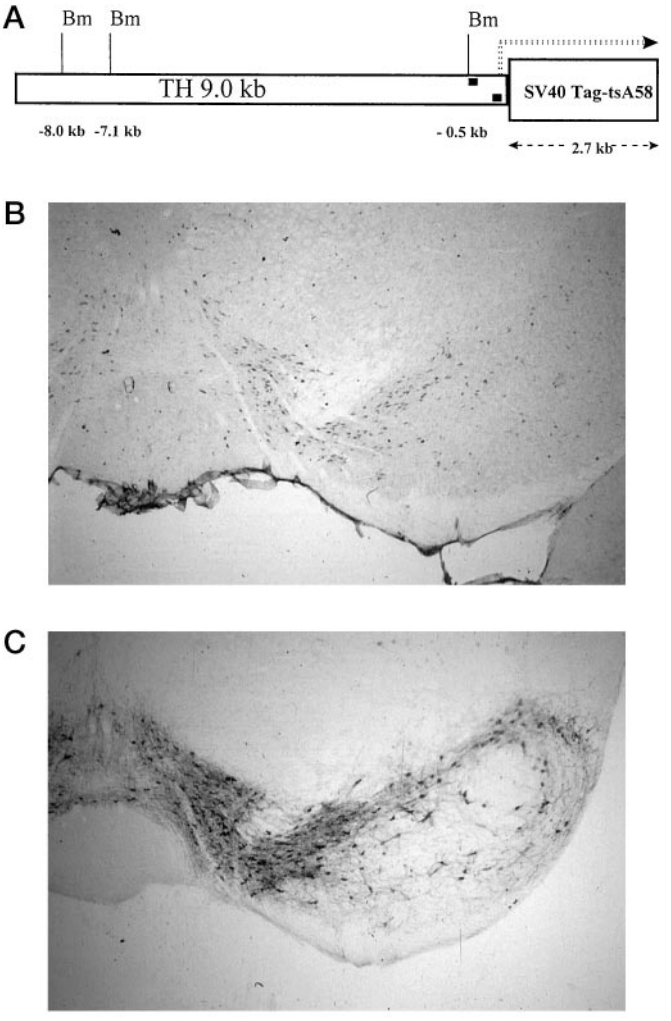


*Figure 1.* The TH9.0 kb-SV40TagA58 hybrid gene and its tissue-specific expression in DA neurons of the transgenic mouse SN. *A*, Diagram of the TH9.0-SV40Tag-tsA58 DNA construct containing 9.0 kb of rat TH 5′ promoter region, the transcription initiation site (*dashed lines*), and 27 bp of 5′ untranslated region fused to a 2.7 kb *Bgl*I/*Bam*HI fragment of SV40-Tag-tsA58. The resulting 11.7 kb of fusion gene was isolated from the plasmid vector and used to generate two transgenic founders, TA58-#8 and TA58-#13. The locations of primers for PCR are marked by *black squares*. *Bm* denotes the restriction enzyme sites for *Bam*HI. *B*, Line TA58-#8 expressed the SV40Tag-A58 in DA neurons of the SN as demonstrated by SV40Tag immunostaining. *C*, TH immunostaining in the adjacent coronal SN sections. The DA neurons are specifically immunostained by both antibodies. Because of the nuclear localization of SV40Tag, the monoclonal SV40Tag antibody did not stain TH-positive fibers, and the intensity of SV40Tag immunostaining was not as strong as TH immunostaining in brain tissue sections.

were coated with polyornithine (1 mg/ml of 0.15 M boric acid buffer, pH 8.4) overnight at 33°C and washed three times with D-PBS before use. Fresh polyornithine solution was made every week. The doubling time for the growth of SN DA cell line was ~36 hr. The cultivation temperature was shifted to nonpermissive temperature (39°C) in either RF medium containing 0.5–1% FCS or Neurobasal medium supplemented with N2 (Life Technologies). For the coculture with primary mesencephalic culture, pooled ventral mesencephalons from 20 embryos (E13.5 for mouse) were collected, trypsinized in $Ca^{2+}/Mg^{2+}$-free D-MEM containing 0.1% trypsin and 0.02% EDTA, transferred to $Ca^{2+}/Mg^{2+}$-free HBSS containing 10% FBS, and gently triturated several times. Then, cells were pelleted by centrifugation at 400 × *g* and resuspended in RF. Viable cells from the 20 embryos were plated in three two-well chamber slides and incubated at 37°C in a 5% $CO_2$ incubator. After 16 hr, cells were rinsed three times with serum-free MEM/F-12 (1:1, v/v) and cultured for 4–8 d in modified N2 medium consisting of MEM/F-12 (1:1, v/v) supplemented with glucose (5 gm/l), HEPES (10 mM), glutamine (220 mg/l), putrescine (10 mM), transferrin (50 μg/ml), Na-selenite (30 nM), insulin (5 μg/ml), progesterone (2 nM), triidothyronine (0.5 nM) or N2 supplement (Life Technologies), and penicillin-streptomycin. The SN4741 cells (1–5 × $10^4$ cells per well) were added to the primary mesencephalic culture after 48 hr and incubated at 37°C for another 48 hr before analysis.

*Immunocytochemistry.* For slide immunocytochemistry (ICC), SN4741 cells were cultured on Lab-Tek chamber slides and fixed for 30 min with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. After washing in 0.1 M PBS containing 0.5% BSA, cultures were incubated overnight with an appropriate antibody. The primary antibodies used were TH, aromatic L-amino acid decarboxylase (AADC) (Eugene Tech), GTP cyclohydrolase (GTPCH) (Hwang et al., 1998), SV40Tag, receptor tyrosine kinase B (TrkB), (Santa Cruz Biotechnology, Santa Cruz, CA), intermediate neurofilament (NF-M), MAP1, MAP2 and synaptophysin (Sigma, St. Louis, MO), neuron-specific enolase (NSE) (Boehringer Mannheim, Indianapolis, IN), and anti-phosphotyrosine (p-Tyr) (Upstate Biotechnology, Lake Placid, NY). Subsequently, slides were incubated for 1 hr with biotinylated rabbit, mouse, or goat IgG (Vector Laboratories, Burlingame, CA), washed twice in 0.1 M PB containing 0.5% BSA and treated with a Vectastain kit as previously described (Son et al., 1996a). The antigens were visualized with 3,3′-diaminobenzidine-HCl (50 mg/ml) and 0.003% $H_2O_2$ as a chromogen. The slide chamber was removed, and cells were dehydrated through graded ethanols and coverslipped with Permount (Fisher Scientific, Houston, TX). TH immunoreactivity of the SN4741 cells was quantified by digitizing dark-field images (720 × 540 μm) of 10 different randomly selected areas of each slide and analyzed as described before (Jahng et al., 1998). For tissues, the ICC procedure was described in detail previously (Min et al., 1994).

*Western blot analysis.* SN4741 cells grown under various experimental conditions were washed twice with 1× PBS, lysed by adding 600 μl (per 100 mm plate) of RIPA buffer containing 1% NP-40, 0.5% Na deoxycholate, 0.1% SDS, PMSF (100 μg/ml), aprotinin (30 μl/ml, Sigma) and Na orthovanadate (1 mM). The cells were scraped off the plate, transferred to a microfuge tube, and passed through a 21 gauge needle to shear DNA and reduce the viscosity. Ten microliters of 10 mg/ml PMSF were added, and continued incubation for 60 min on ice. Cell lysates were microfuged at 15,000 × *g* for 20 min at 4°C. The supernatants were used as the total cell lysates. Total mouse brain extract was similarly prepared from an 8-week-old F1 hybrid female. A431 cell extract was obtained from Upstate Biotechnology (Lake Placid, NY). Cell lysate samples were heated to 95–100°C for 5 min, cooled on ice, microfuged for 5 min, and 20 μl were loaded onto SDS-PAGE gel (10 × 10 cm). Protein concentration was determined by the Lowry method (Lowry et al., 1951). Electrotransfer to nitrocellulose membrane was performed in a 8% SDS-PAGE gel (10–20 μg/lane) at 15 V/cm for 1 hr. After transfer, the membrane was incubated in 25 ml blocking buffer (1× TBS, 0.1% Tween 20 with 5% w/v nonfat dry milk) for 1–3 hr at room temperature and incubated with a primary antibody (at appropriate dilution) in 5 ml primary antibody dilution buffer (1× TBS, 0.1% Tween 20 with 0.5% nonfat dry milk) overnight at 4°C. The membrane was washed three times for 10 min each with an excess volume of TBST (1× TBS, 0.1% Tween 20) and incubated with HRP-conjugated secondary antibody (1:2000) in 5 ml of the antibody dilution buffer for 1 hr at room temperature. After washing 3 times for 10 min each with an excess volume of TBST, the proteins were detected by ECL chemiluminescence assay method (Amersham, Arlington Heights, IL). The bands recognized by the primary antibody were visualized by exposure to x-ray film.

*RT-PCR.* Total RNA from each SN4741 cell culture was isolated by homogenization in 800 μl of RNAzol-B (Cina-Biotecz, Houston, TX). For RT-PCR, 1–2 μg of total RNA was reverse transcribed in 20 μl of reaction solution containing 1 × PCR buffer, 2.5 mM $MgCl_2$, 0.5 mM dNTPs, and 200 U of Moloney murine leukemia virus reverse transcriptase in the presence of [$^{32}$P]dCTP and oligo-dT, as recommended by the manufacturer (Life Technologies). The resulting cDNA was quantified by determining the amount of radioactivity incorporated into trichloroacetic acid-precipitable nucleic acid. PCR was performed with *Taq* polymerase (Perkin-Elmer, Norwalk, CT) using an appropriate primer sets for BDNF (nucleotides 374–393 and 761–780; GenBank accession number G287898), neurotrophin-3 (NT-3, nucleotides 441–460 and 851–870; GenBank accession number X53257), $D_2$ autoreceptor ($D_2$R, nucleotides 1581–1600 and 2051–2070; GenBank accession number X55674), and dopamine transporter (DA-T, nucleotides 2381–2400 and 6241–6250; GenBank Accession number U15791), a β-actin internal standard for normalization (Gene Link, Thornwood, NY), trace amounts of [$^{32}$P]dCTP, and various amounts of cDNA. Samples (1 and 5 μl) were run on 1% agarose gel and transferred to nitrocellulose. Blots were hybridized with radiolabeled human BDNF cDNA (Dr. Howard Federoff, University of Rochester, Rochester, NY), rat NT-3 cDNA (Regeneron Pharmaceutical, Tarrytown, NY), mouse DA-T cDNA (Dr. Joe Cubells, Yale University, New Haven, CT), or mouse $D_2$R oligonucleotide (nucleotides 1801–1850) probe, respectively. Also, these major PCR bands were cloned into the pGEM-T vector (Promega, Madison, WI) and confirmed their identity by DNA sequence determination.

*Quantitative measurement of [$^3$H]dopamine uptake and dopamine synthesis.* SN4741 cell cultures were incubated at 33°C (if necessary, at 39°C) in DMEM supplemented with 5 × $10^{-8}$ M [$^3$H]dopamine (Amersham; specific activity 44–49 Ci/mmol) and 100 μM ascorbic acid. As a control for nonspecific dopamine accumulation, sister cultures were incubated with [$^3$H]dopamine solution with 10 μM mazindol (dopamine uptake blocker). When uptake reached saturation, usually 15 min, the incubation was terminated by rinsing the cells three times with PBS. Then, the cells were lysed in 0.1% SDS and radioactivity associated with cells was measured by liquid scintillation counter. The specific dopamine uptake (femtomoles per DA cell) was defined as the difference between the uptake in the presence of mazindol and the uptake without mazindol. Dopamine was extracted by perchloric acid from SN4741 cell pellets grown in 33°C in RF medium and quantitated by HPLC using a C18 column (Beckman Instruments, Fullerton, CA) equipped with an electrochemical detector (Waters Corporation, Milford, MA).

*Measurement of BDNF release by ELISA.* For collection of conditioned media, 5 × $10^5$ cells per well were seeded onto six-well plates in RF medium and allowed to attach for 24 hr. Media were replaced with 1 ml of MEM/F-12 (1:1) containing N2 in the presence of 5 μM $MPP^+$, 0.05 mM sodium nitroprusside (SNP), 0.05 mM $H_2O_2$, and 0.1 mM glutamate or TNF-α (50 ng/ml), and plates were placed at 33°C in 5% $CO_2$. Conditioned media were collected after 24 hr and stored at −80°C until assayed by ELISA. Each treatment was performed in triplicate. The amount of BDNF in each cell-conditioned medium was quantified by using BDNF Emax ImmunoAssay system (Promega) as suggested by the company.

*Pharmacological treatment with $MPP^+$, glutamate, SNP, dopamine, and BDNF.* The treatment conditions for $MPP^+$, glutamate, SNP, dopamine, and BDNF were adopted from the previously reported conditions for the primary mesencephalic and brain neuronal cultures (Hyman et al., 1991; Dawson et al., 1993). Briefly, the SN4741 cells were plated at a density of 2–3 × $10^5$ cells per well on 6 cm plates in RF medium. To evaluate the protective effect of BDNF, cells were stabilized for 24 hr in RF medium after achieving ~35% confluence and replaced with RF medium (1% FCS) containing either 10 μM $MPP^+$ (Research Biochemicals, Natick, MA) only or 10 μM $MPP^+$ with 50 ng/ml BDNF and kept for a further 24 hr. In some cultures, 50 ng/ml mouse NGF was included instead of BDNF (Genzyme, Cambridge, MA) as a negative control. The recovery or protection against $MPP^+$ treatment was measured by the total cell counting using trypan blue dye exclusion. To measure SNP-, glutamate-, or dopamine-mediated neurotoxicity the SN4741 cells were pretreated with BDNF (50 ng/ml) for 10 min and exposed to either 0.5 mM SNP for 5 min, 0.5 mM glutamate for 10 min, or 5–15 nM dopamine for 18 hr. Then the cells were transferred to RF medium (1% FCS) with or without BDNF and kept for a further 24 hr before total cell counting using trypan blue dye exclusion. In a single experiment each treatment was performed in quadruplicate.

*Statistical analysis.* The results obtained from three or four different

plates were expressed as the mean ± SEM. The same experiment was repeated at least three times. The data were analyzed using GraphPad Prism data analysis program (GraphPad Software, San Diego, CA). For the comparison of statistical significance between two groups, Student's *t* tests for paired and unpaired data were used. For multiple comparison, one-way ANOVA followed by *post hoc* comparisons of the group means according to the method of Tukey was used. $p$ values $< 0.05$ were considered significant.

## RESULTS

### The conditional immortalization of DA cells of embryonic SN origin

A DA neuronal cell line was established by targeted immortalization in transgenic mouse embryos, in which the high level expression of the temperature-sensitive mutant form of an oncogene, SV40Tag-tsA58, was directed to brain DA neuron. Previously, we have demonstrated that a 9.0 kb of 5′ upstream promoter region of the rat TH gene can direct expression of lacZ reporter gene limited to DA neurons in both developing and adult transgenic mice (Min et al., 1994; Son et al., 1996b). Therefore, we first constructed the hybrid gene, *TH9.0-SV40Tag-tsA58*, consisting of 9.0 kb of 5′ TH promoter region fused to the modulatable oncogene, SV40Tag-tsA58 (Fig. 1*A*) and directed DA neuron-specific expression of the thermolabile SV40Tag in transgenic mice. The DA cell type-specific expression of the SV40Tag-tsA58 in adult mouse brain provided us with a tool to isolate DA neuronal progenitor cells from the embryonic SN tissue at an early developmental stage for further immortalization *in vitro*. Three independent transgenic founders were identified by the genomic Southern blot analysis. All three founders had 2–20 copies of the transgene per diploid genome. Among them line TA58-#8 consistently produced progeny that expressed the SV40Tag-A58 in DA neurons of the SN and VTA as shown by SV40Tag immunostaining (Fig. 1*B*) and TH immunostaining (Fig. 1*C*) in the adjacent brain sections. Because of the nuclear localization of SV40Tag, the monoclonal SV40Tag antibody did not stain TH-positive fibers, and the intensity of SV40Tag immunostaining was not as strong as TH immunostaining in brain tissue sections. Progeny of line TA58-#8 did not develop any obvious tumors in adulthood (between 4 and 6 months of age). Thus, the transgenic mouse line TA58-#8 was used for further isolation of the immortalized DA cell lines during embryonic development.

For the establishment of immortalized SN DA cell lines, the E13.5 transgenic embryonic brains from line TA58-#8 were used as a primary source of SN DA progenitor cells expressing the SV40Tag-tsA58. In E13.5 embryonic mouse brain, the mesencephalic TH-positive DA progenitor and/or immature DA neurons were densely clustered over the mesencephalic flexure, demonstrated from our previous ontogenic study using the TH9.0-lacZ transgenic embryos (Son et al., 1996b). In addition, at this stage the mesencephalon appeared to have no obvious transient TH-positive cells, which could be a potential source of contamination during the selection procedure in immortalized SN-derived DA progenitor cells. Therefore, for the isolation of immortalized SN-derived DA progenitor cells, the dissected embryonic SN cells from the E13.5 transgenic embryos were cultured in RF medium at the permissive temperature, 33°C for 3–8 weeks until distinct colonies were observed. The terminally differentiated primary DA neurons usually died within a week and never formed colonies. The glial cells from SN regions neither formed colonies nor survived beyond 3–4 weeks in our culture condition. The clonal origin of an immortalized colony was monitored by regular microscopic observation during its formation, as exemplified in Figure 2*A*. After propagation of each colony, expression of TH and SV40Tag-A58 was confirmed by the immunostaining during the entire cloning procedure. After the third cloning step, the SN DA cell line became morphologically flatter compared with the early clones (Fig. 2, compare *A*, *B*). As shown in Figure 2, *B* and *C*, a representative SN DA cell line SN4741 was stained with TH antibody and more intensely with SV40Tag antibody, respectively. The TH antibody stained the cytoplasm, whereas SV40Tag antibody stained exclusively the nucleus. The SN4741 cell line showed the persistent expression of the marker enzyme TH and the oncogene SV40Tag-A58 at the permissive temperature, 33°C. The morphology of SN4741 cells showed the relatively consistent shapes when grown on a polyornithine-coated slide, but the morphology became more or less heterogeneous at the high confluence on the uncoated plastic culture dishes as passages increased. This may be caused by progressive differentiation induced by autocrine action of various endogenously expressed neurotrophins and cytokines (our unpublished results). Thus, morphological differentiation was monitored very carefully while maintaining the SN4741 cells, particularly in the high-density cultures.

### Expression of the known DA neuronal markers in the SN DA cell line

The SN DA progenitor cell line SN4741 was further characterized at the permissive temperature (33°C) for the expression of general neuronal markers, specific DA neuronal markers, neurotrophins and their receptors by immnohistochemistry, Western blot analysis, RT-PCR, or biochemical assay. Immunohistochemical analysis indicated the presence of neuron-specific markers, such as NF-M, NSE, MAP1, MAP2, synaptophysin, and DA neuron-specific markers, such as TH, AADC, and GTPCH (Table 1). In particular, the representative DA marker TH expression was confirmed by Western blot analysis after maintaining the SN4741 cells for 1 week in high-density culture (Fig. 3*A*). The 62 kDa TH band in the SN4741 cell line was consistent with the molecular weight of TH isolated from mouse adrenal gland and similarly derived locus coeruleus noradrenergic cell line, which were used as positive controls for the Western blot analysis. The specific amount of TH protein in the cell line grown at 37°C was higher than the culture at 33°C. As demonstrated in a TH-positive pheochromocytoma cell, PC12 line (Kim et al., 1995), the SN4741 cells also exhibited increased TH expression at the high cell density during a prolonged culture (7–10 d). AADC, the second enzyme for the biosynthesis of dopamine, was also detected by immunocytochemistry at a low level. But GTPCH, an enzyme for the biosynthesis of cofactor biopterin, was expressed at a moderate level (Table 1). Because all the necessary enzymes for the biosynthesis of dopamine were expressed, dopamine content of the SN4741 cells was measured in cellular extracts by a reverse-phase HPLC method. The level of dopamine was 4 pmol/mg protein, which could be increased twofold to fourfold by enhancing the TH expression in the prolonged culture (7–10 d). The expression of other DA neuron-specific markers, such as NT-3, BDNF, DA-T, and $D_2R$ were detected by RT-PCR in the SN4741 cells (Fig. 3*B*). NT-3 and BDNF were expressed at much higher levels than DA-T or $D_2R$ ($p < 0.05$). The expected major band sizes were 430 bp for NT-3, 407 bp for BDNF, 553 bp for DA-T, and 490 bp for $D_2R$. After Southern blotting of both 1 and 5 $\mu$l of each RT-PCR sample, the presence of each specific size band (the strongest band) was detected by the specific cDNA probe, and its

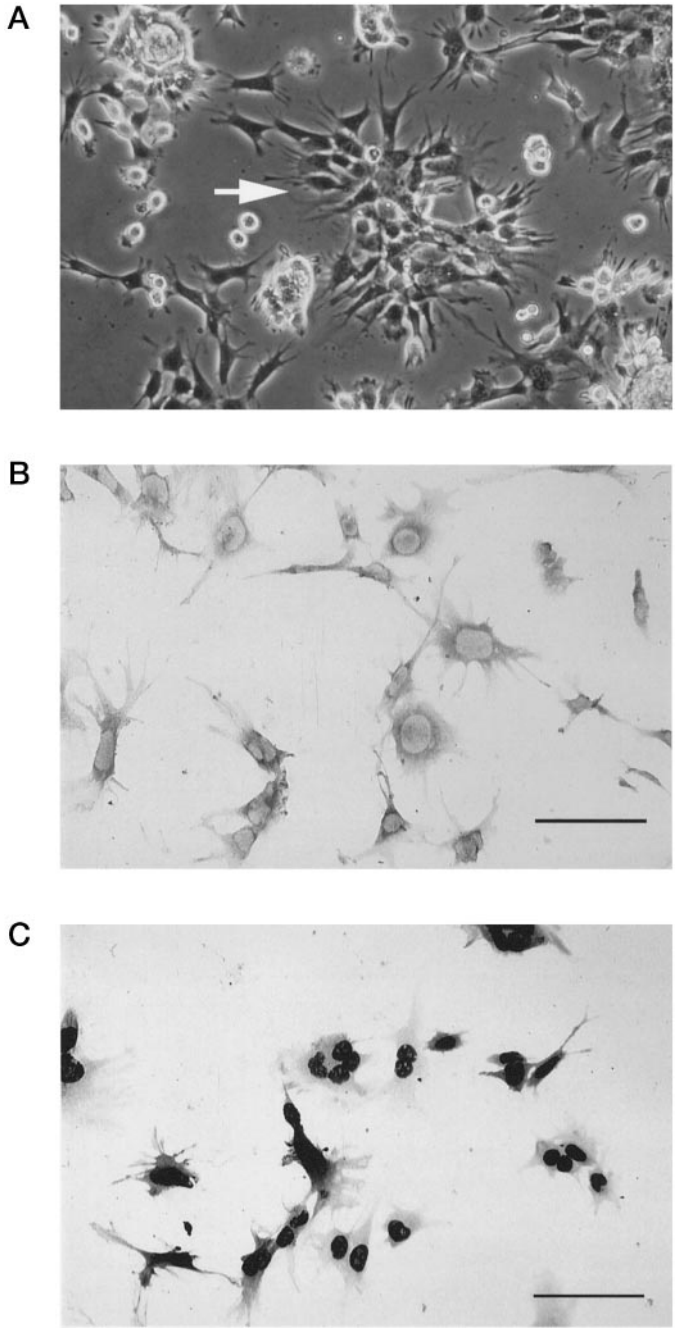


*Figure 2.* TH and SV40Tag immunostaining of SN DA cell line SN4741. For the establishment of DA cell lines, mesencephalic SN regions from E13.5 embryos from TA58-#8 were surgically removed under sterile conditions. The collected SN tissues were dissociated and cultured at 33°C with 5% $CO_2$ in RF medium. The dispersed primary neuronal cell lines from the transgenic embryos were grown through repeated passages for 3–4 months to establish a pure cell population. The clonal origin of a colony was monitored and confirmed by microscopic observation during its colony formation. *A*, The morphology of an early SN DA cell colony (*arrow*) named SN-47. After the third cloning step, the DA cell line became morphologically flatter compared with the early clones. A representative SN DA cell line SN4741 was immunostained with TH polyclonal antibody (*B*) and more intensely with SV40Tag monoclonal antibody (*C*), respectively. The TH antibody stained the cytoplasm, whereas SV40Tag antibody stained exclusively the nucleus. Scale bars: *B*, *C*, 100 μm.

**Table 1. Comparison of the expression of various DA neuronal markers under permissive (33°C) and nonpermissive temperature (39°C) in the SN4741 cells**

| Markers | 33°C | 39°C |
|---|---|---|
| TH | + | + |
| AADC | + | + |
| GTPCH | ++ | ++ |
| NF-M | + | ++ |
| NSE | + | + |
| MAP1 | + | ++ |
| MAP2 | ++ | +++ |
| Synaptophysin | + | + |
| SV40 Tag | +++ | + |
| BDNF | ++ | + |
| NT-3 | ++ | + |
| TrkB | + | + |
| $D_2R$ | + | + |
| DA-T | + | ++ |

The expression of general neuronal markers, such as NSE, MAP1, MAP2, NF-M, and synaptophysin, and specific DA neuronal markers, such as TH, AADC, and GTPCH, were determined by immunocytochemistry. The expression of DA-T, $D_2R$, BDNF, and NT-3 were semiquantitated by RT-PCR. DA-T was also determined by dopamine uptake. TH and TrkB expressions were confirmed by Western blot analysis. Note: +, low; ++, medium; +++, high.

identity was confirmed by the determination of DNA sequence as described in Materials and Methods. The SN4741 cell line, morphologically differentiated at the nonpermissive temperature (39°C), showed enhanced expression of MAP1, MAP2, NF-M, synaptophysin, and DA-T (see next section for details). The DA-T expression was further determined by quantitative measurement of [$^3$H]dopamine uptake in the presence of 100 mM ascorbic acid. The dopamine uptake rate was 150 fmol/mg protein per 15 min in the SN4741 cells grown at 33°C in RF medium. The dopamine uptake rate increased approximately twofold after phenotypic differentiation at 39°C.

## Morphological and phenotypic differentiation of the SN DA cell line

When grown at 33°C, the SN4741 cells proliferated with a doubling time of 36 hr, having a fibroblast-like flat morphology with less prominent neurite growth, as shown in Figure 4*A*. As described above, the SN4741 cells were immunopositive for various neuronal markers, but the representative DA phenotype marker TH was much lower than that in the differentiated DA neurons (Fig. 4*E*, *arrow*) of the primary mesencephalic culture (Fig. 4, compare *D* with the *arrow* in *E*). When the SN4741 cells were shifted to a differentiation condition (i.e., nonpermissive temperature 39°C and reduction of FCS to 0.5%), the cells ceased proliferation and, after 2 d, started to display a neuronal morphology with extensive neurite outgrowth and long bipolar or multipolar processes (Fig. 4*B*). Under these conditions nuclear SV40Tag staining was greatly reduced, but MAP1 and MAP2 immunoreactivities were markedly increased, and expression of NT-3 and BDNF mRNAs was decreased, determined by semiquantitative RT-PCR. Dopamine uptake was increased twofold ($p < 0.05$) when measured by the uptake of $^3$H-labeled dopamine (Table 1). However, the representative phenotype marker, TH immunoreactivity, was not enhanced at the nonpermissive temperature, 39°C, contrary to expectation. The data suggests that the existence of unknown factors in the primary embryonic mesencephalic cultures might be required for further phenotypic

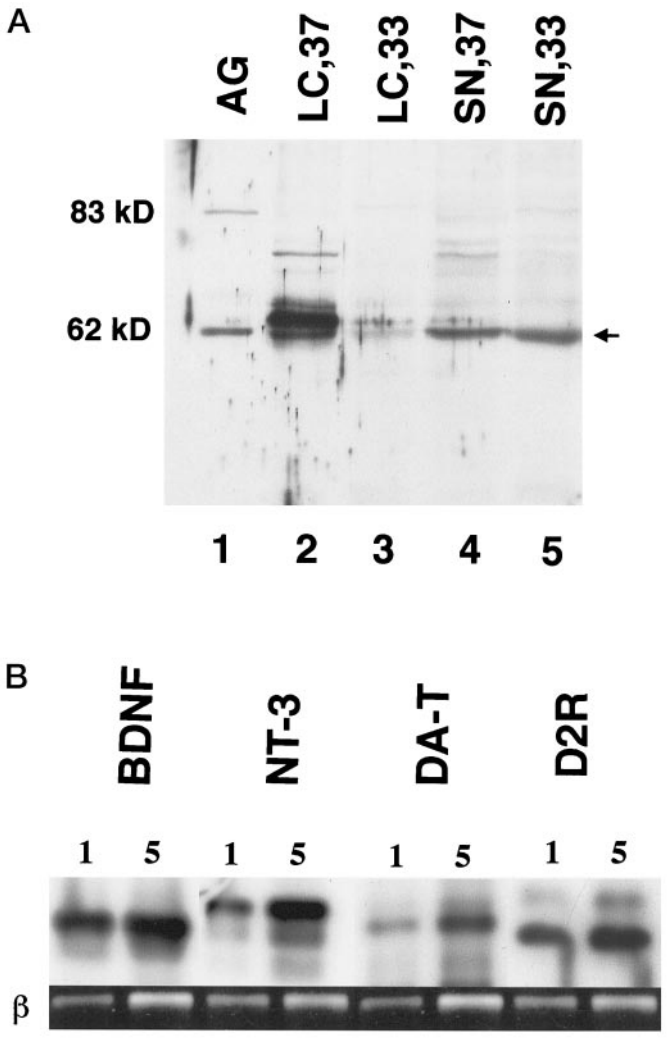


*Figure 3.* Detection of TH expression and DA neuronal markers by Western blot analysis and RT-PCR. *A*, The representative DA marker, TH expression, was confirmed by Western blot analysis after maintaining the SN4741 cells for a week in high-density culture at 37°C (*lane 4*) and 33°C (*lane 5*). The 62 kDa TH band in the SN4741 cells was consistent with the molecular weight of TH isolated from mouse adrenal gland (*lane 1*) and similarly derived locus coeruleus noradrenergic cell line, grown at 37°C (*lane 2*) and 33°C (*lane 3*), which were used as positive controls for the Western blot analysis. Each lane contained 15 $\mu$g of protein except lane 1 (5 $\mu$g) and lane 2 (30 $\mu$g). The specific amount of TH protein in the SN4741 cells grown at 37°C was higher than the culture at 33°C. As demonstrated in a TH-positive pheochromocytoma cell, PC12, culture (Kim et al., 1995), our SN DA cell line also exhibited the increase in TH expression in the high cell density during a prolonged culture (7–10 d). *B*, The expression of some DA neuron-specific markers, such as NT-3, BDNF, DA-T, and $D_2R$ were detected by RT-PCR in the SN4741 cell line. After Southern blotting of both 1 and 5 $\mu$l of each RT-PCR sample, the presence of each specific size band (the strongest band) was detected and confirmed with radiolabeled human BDNF cDNA, rat NT-3 cDNA, mouse DA-T cDNA, or mouse $D_2R$ oligonucleotide probe, respectively. Each filter was hybridized with each specific probe and exposed separately. The expected major band sizes were 430 bp for NT-3, 407 bp for BDNF, 553 bp for DA-T, and 490 bp for $D_2R$. $\beta$ represents the $\beta$-actin PCR product of 289 bp as an internal standard.

differentiation of the SN4741 cell line including TH expression. To test this hypothesis, the SN4741 cells were cocultured with primary embryonic mesencephalic cells derived from E13.5 mouse embryos. Indeed, as expected, the TH immunoreactivity of most SN4741 cells was greatly enhanced (fivefold to sevenfold) in the presence of the mesencephalic culture (Fig. 4, compare *D*, *E*; $p < 0.05$). In the coculture slide, the SN4741 cells can be distinguished from the primary DA neurons by their distinct morphology and size (Fig. 4*E*). The primary DA neurons (*arrow*) were morphologically multipolar or bipolar and much smaller than SN4741 cells. Next, we tested whether the retinoic acid affected the morphological and phenotypic differentiation of the SN4741 cell line. Although both the retinoic acid receptor (Krezel et al., 1998) and an orphan nuclear receptor Nurr1, being activated through heterodimerization with retinoic acid receptor, (Zetterstrom et al., 1997) were known to be critically involved in the DA neuronal development, the presence of retinoic acid did not significantly enhance the TH immunostaining during the morphological differentiation at 39°C. Retinoic acid changed their morphology causing much longer and bipolar neurite extensions and consistently higher MAP2 expression (Fig. 4*C*,*F*).

### $MPP^+$-induced cell death, its protection by BDNF, and autocrine production of BDNF in SN DA cell line

Based on the above phenotypic characterization, we further investigated the pharmacological aspect of the SN4741 cell line using several experimental neurotoxicity paradigms known to produce DA neuronal degeneration, such as exposure to the neurotoxin $MPP^+$, the excitatory amino acid glutamate, NO, and dopamine. Exposure of the SN4741 cell line to 10 $\mu$M $MPP^+$ for 15 hr, 0.5 mM glutamate for 10 min, 0.5 mM SNP (an NO donor) for 5 min or 5–15 nM dopamine for 18 hr resulted in a loss of >45–55% ($p < 0.05$) of the plated SN4741 cells during overnight culture, which indicates our SN4741 cells are as sensitive as DA neurons in primary mesencephalic culture to $MPP^+$-, glutamate-, NO-, or dopamine-induced neurotoxicity (Fig. 5). The neuroprotective role of BDNF was also examined in the SN4741 cell line under the above described neurotoxicity paradigms. In contrast, BDNF demonstrated the significant neuroprotection against $MPP^+$-, glutamate-, or NO-induced neurotoxicity by reducing the loss of SN4741 cells to <15–29% ($p < 0.05$), but not against dopamine-induced neurotoxicity. In control cell cultures BDNF did not affect cell growth and total cell number. The neuroprotective functions of BDNF was further tested in comparison with NGF against $MPP^+$-induced neurotoxicity in the homogeneous SN4741 cell culture. NGF was known not to promote the survival of mesencephalic DA neurons *in vitro* in contrast to BDNF (Hyman et al., 1991). Intriguingly, when SN4741 cultures were treated with BDNF (50 ng/ml) after exposure to $MPP^+$, the total cell loss of the SN4741 culture was significantly reduced to <16% ($p < 0.05$) in contrast to NGF treatment showing 46% ($p < 0.05$) of the cell loss which exerted no protection effect at all (Fig. 6). Pretreatment of the SN4741 cells with BDNF before exposure to $MPP^+$ gave a slightly better protection (5% less cell death; $p < 0.05$) than no pretreatment in its protective efficacy against $MPP^+$ damage. This result suggests that the SN DA cell line SN4741 will be useful *in vitro* model system of DA neurons in SN.

The ELISA assays showed that the SN4741 cells released 200 pg of BDNF per day per $4 \times 10^5$ cells under unstressed conditions, which may constitutively activate intrinsic TrkB receptors in high-density culture (see Fig. 8). To test whether autocrine/paracrine release of BDNF is affected by the experimental neurotoxicity paradigms as a self-defensive neuroprotective mechanism, cultures were treated with a sublethal dose of the various neurotoxic stresses. Because TrkB is expressed in the SN4741 cell

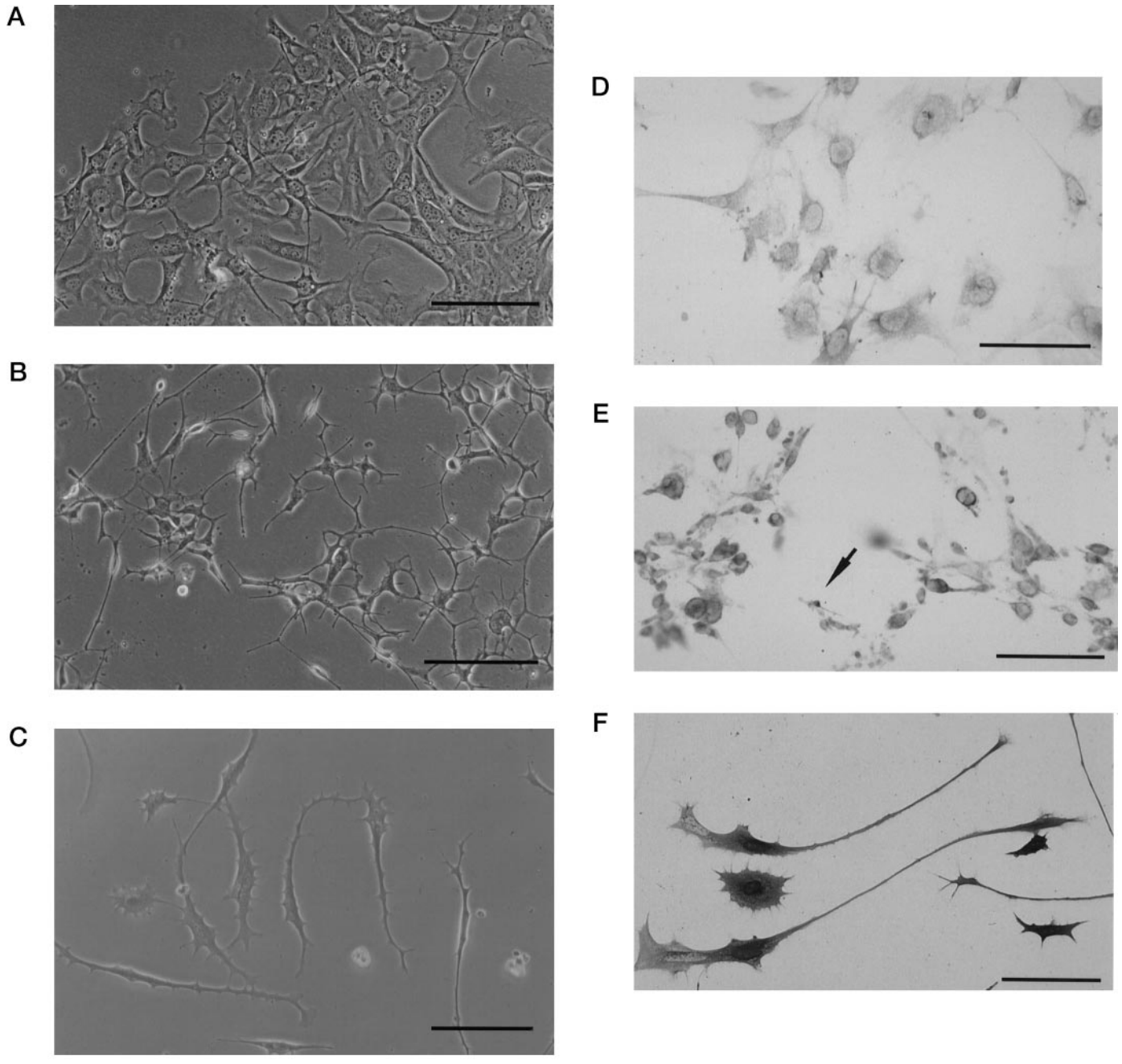


*Figure 4.* Morphological and phenotypic differentiation of the SN DA cell line SN4741 under various culture conditions. The morphological differentiation of the SN4741 cells was induced by culturing at the nonpermissive temperature (39°C) with a minimal serum concentration and/or retinoic acid. *A*, When grown at 33°C, the SN4741 cells have a fibroblast-like flat morphology with less prominent neurite growth. *B*, Under differentiation condition (i.e., nonpermissive temperature 39°C and reduction of FCS to 0.5%), the SN4741 cell line ceased proliferation and, after 2 d, started to display a neuronal morphology with extensive neurite outgrowth and, after 4 d, long bipolar or multipolar processes. *C*, Retinoic acid caused much longer and bipolar neurite extensions at 39°C. In contrast to the control SN4741 culture (*D*), the TH immunoreactivity of most SN4741 cell lines was greatly enhanced in the presence of the mesencephalic culture (*E*). In the coculture slides, the SN4741 cells can be distinguished from the primary DA neurons by their distinct morphology and size. The primary DA neurons (*arrow*) were morphologically multipolar or bipolar and much smaller than the SN4741 cells. *F*, MAP2 expression became consistently higher in the presence of retinoic acid at 39°C. Scale bars: *A–C*, 280 μm; *D–F*, 140 μm.

line, the secretion of BDNF was simply measured by ELISA under the neurotoxic stresses. As demonstrated in Figure 7, the NO donor SNP and free-radical donor $H_2O_2$ inhibited the BDNF release significantly (30–40%; $p < 0.05$) but not $MPP^+$. In contrast, glutamate and a proinflammatory cytokine, TNF-α enhanced the BDNF release by 30–50% ($p < 0.05$) in the culture conditions used.

### Activation of TrkB by BDNF treatment in SN4741 cell line

BDNF exerts its biological functions by activation of a cascade of intracellular signaling pathway(s), mediated by the intracellular tyrosine kinase domain of the TrkB receptor. Activation of TrkB via the phosphorylation of a tyrosine residue by BDNF is crucial to trigger the signal transduction pathway(s) required for the DA

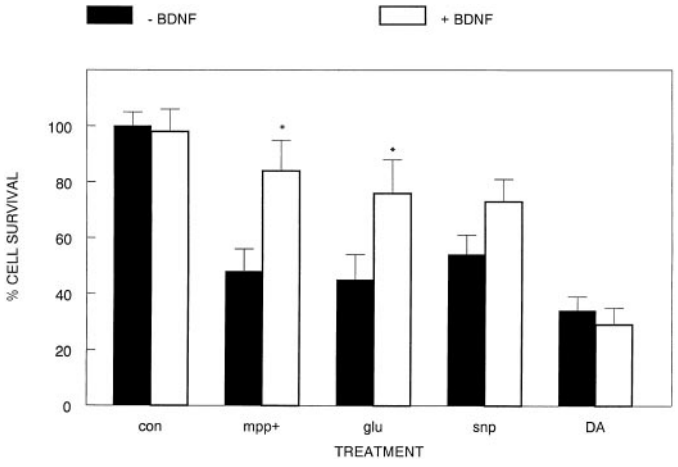


*Figure 5.* BDNF protected the SN4741 cell line against various neurotoxic stresses. To measure BDNF-mediated neuroprotection against $MPP^+$-, SNP-, glutamate-, or dopamine-mediated neurotoxicity, the SN4741 cells were pretreated with BDNF (50 ng/ml) for 10 min (*open bars*) and exposed to either 10 $\mu$M $MPP^+$ for 15 hr, 0.5 mM glutamate for 10 min, 0.5 mM SNP for 5 min, or 5–15 nM dopamine for 18 hr in the presence of BDNF (*open bars*). *Solid bars* represents each control group. BDNF protected against $MPP^+$-, glutamate-, or NO-induced neurotoxicity, but not against dopamine-induced neurotoxicity. All values are the mean ± SEM; $^*p < 0.05$.

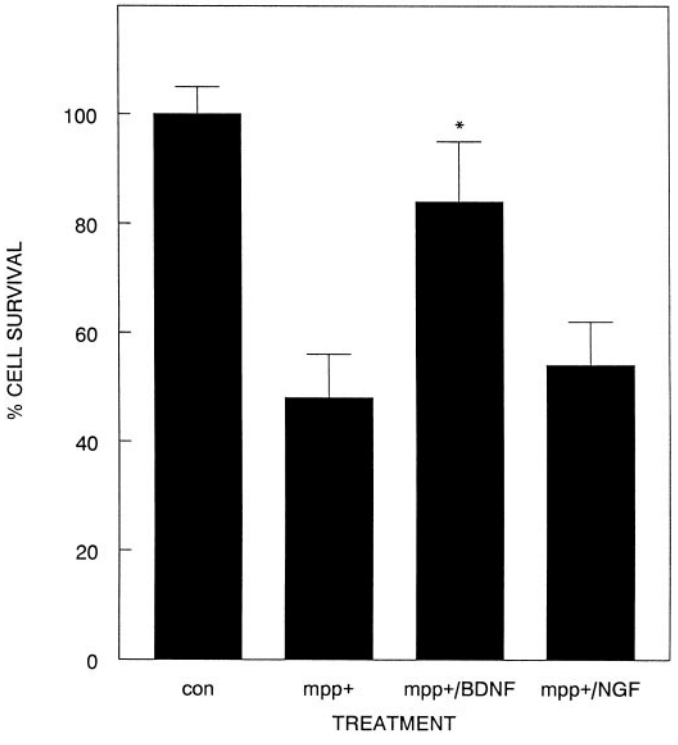


*Figure 6.* BDNF but not NGF protects against $MPP^+$-induced neurotoxicity. The neuroprotective functions of BDNF were further tested in comparison with NGF against $MPP^+$-induced neurotoxicity in the SN DA cell culture. Only BDNF exerted its neuroprotective function against $MPP^+$-neurotoxicity. All values are the mean ± SEM; $^*p < 0.05$.

neuroprotection under both normal and stress conditions. After demonstrating that BDNF can rescue $MPP^+$-induced neurotoxicity, and BDNF secretion can be modulated by the stress in the SN4741 cells, it was tested whether either exogenous BDNF or

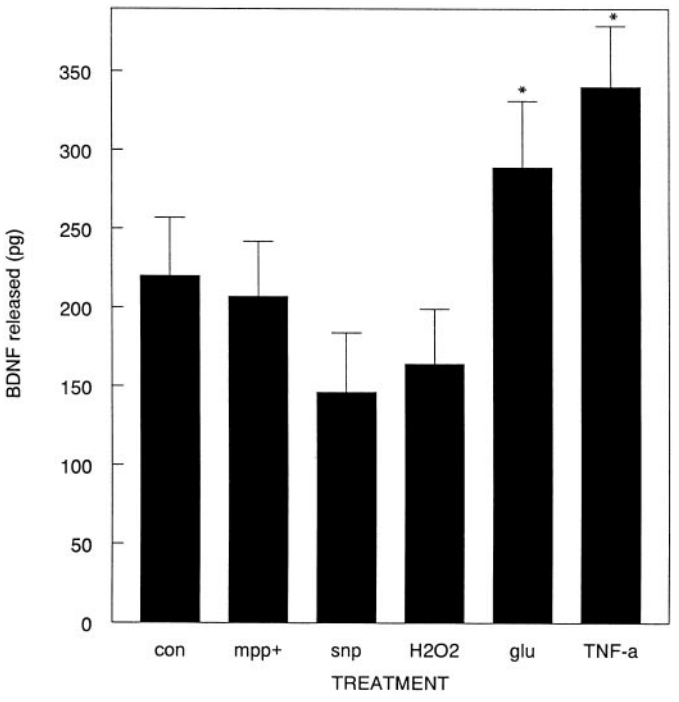


*Figure 7.* BDNF secretion from the SN DA cell line under normal and stress conditions. The five representative classes of known neurotoxic insults, such as mitochondrial electron transport inhibitor ($MPP^+$), free radical generator ($H_2O_2$), NO producer (SNP), excitatory amino acid (glutamate), and proinflammatory cytokine (TNF-$\alpha$) were tested. After treatment with sublethal doses of $MPP^+$ (5 $\mu$M), SNP (0.05 mM), $H_2O_2$ (0.05 mM), glutamate (0.1 mM), or TNF-$\alpha$ (50 ng/ml), the amount of BDNF in the cell-conditioned medium was quantified by ELISA. Glutamate and TNF-$\alpha$ increased the release of BDNF by 30–50%. Of interest, two potential free radical generators, NO (SNP) and $H_2O_2$, but not $MPP^+$, significantly suppressed the amount of BDNF release by ~30–40%. All values are the mean ± SEM; $^*p < 0.05$.

autocrine BDNF induced the activation of TrkB. First, the presence of TrkB in SN4741 cells was demonstrated by Western blot analysis using TrkB antibody (Fig. 8*A*). Total mouse brain extract was used as a positive source of TrkB protein, and A431 cell extract as a negative control as the supplier suggested. As shown in Figure 8*B*, using the same cellular or brain preparations, the exogenous BDNF treatment for 24 hr led to a significant increase of tyrosine phosphorylation of TrkB compared with the non-treated TrkB-expressing SN4741 cells, as detected by Western blot using the p-Tyr antibody. The basal level of TrkB activation, without the exogenous BDNF, was consistently observed at >60–70% confluence, in which the activation of TrkB could be induced by both autocrine and paracrine actions of the secreted BDNF. Consistent with this observation, the sensitivity of SN4741 cell line to $MPP^+$-induced neurotoxicity was highest at the low-density culture (~30–35% confluence), which we adopted for our experimental condition as described in Materials and Methods. Thus, BDNF-induced neuroprotection against $MPP^+$–, glutamate-, or NO-neurotoxicity appears to be mediated through TrkB activation in SN4741 cell line.

## DISCUSSION

This study characterized a unique SN-derived DA progenitor cell line as a model system for the molecular analysis of the BDNF-regulated DA neuroprotection and neuronal differentiation. In particular, the SN DA cell line showed $MPP^+$-induced neuro-

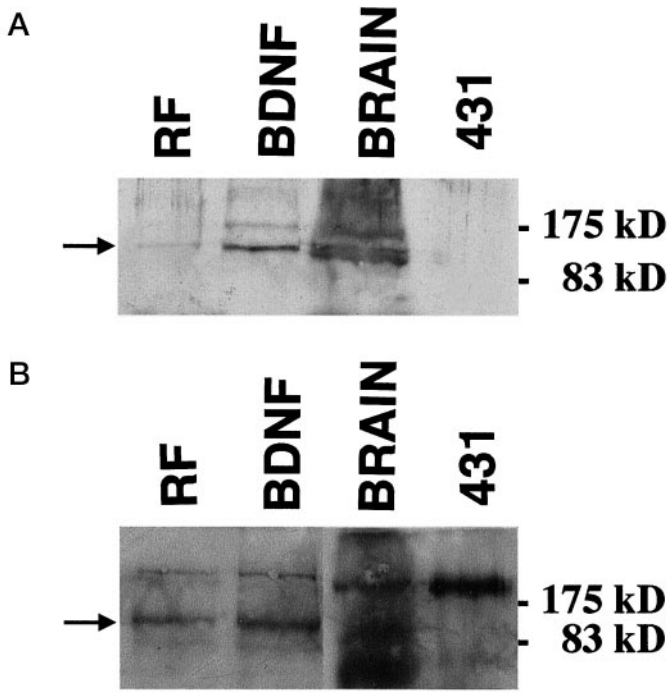


*Figure 8.* Tyrosine phosphorylation of TrkB by BDNF treatment. *A*, The presence of TrkB in SN4741 cell line was demonstrated by Western blot analysis using TrkB antibody. The first lane (*RF*) contains the SN4741 cell lysate grown in RF medium for 2 d, and the second lane (*BDNF*) contains the SN4741 cell lysate grown in RF medium containing BDNF (50 ng/ml) for 24 hr. Total mouse brain extract (*BRAIN*) was used as a positive source of TrkB protein and A431 cell extract (*431*) as a negative control as the supplier suggested. *B*, The exogenous BDNF treatment (*BDNF*) for 24 hr led to a significant increase of tyrosine phosphorylation of TrkB compared with the nontreated TrkB expressing SN4741 cell line (*RF*), as detected by Western blot using the p-Tyr antibody. The first lane (*RF*) contains the SN4741 lysate grown in RF medium for 48 hr, and the second lane (*BDNF*) includes the cell lysate grown in RF medium containing BDNF (50 ng/ml) for 24 hr. A basal level of TrkB activation (*RF*), without the exogenous BDNF, was consistently observed at >60–70% confluence, in which the activation of TrkB could be induced by the secreted BDNF. Total mouse brain extract (*BRAIN*) was used as a positive source of the activated TrkB protein and A431 cell extract (*431*) as a negative control.

toxic cell death and BDNF-evoked neuroprotection similar to that observed in primary mesencephalic culture.

### Immortalized DA neuronal cell line were derived from the SN of transgenic embryos

During mammalian CNS ontogeny the peak appearance of midbrain DA progenitor cells occurs between E9.5 and E10.5 in mice (Di Porzio et al., 1990; Son et al., 1996b). The adult mouse anatomical pattern of DA neuronal distribution emerged at E13.5, accompanying the physical distinction of SN (A9) from VTA (A10). At this stage most DA neurons are postmitotic with a minor population of DA progenitor cells. To avoid the ambiguity in the origin of immortalized DA cell line, two different strategies were adopted using transgenic mice. First, a DA neuron-specific promoter (Min et al., 1994; Son et al., 1996b) was used for the targeted expression of an oncogene, which was able to provide selectivity during the immortalization procedure. As demonstrated by immunohistochemistry, the 9.0 kb of the TH promoter directed very specific expression of the oncogene to DA neurons of SN. Second, a temperature-sensitive oncogene was used for the definitive anatomical origin of an immortalized cell line as well as further manipulation of DA neurons *in vitro*. This strategy allowed us to isolate and immortalize the DA neuronal progenitor cells from embryonic SN at early developmental stages without the significant alteration of their normal genetic content, which often occurs in tumor cell lines obtained from full-blown adult tumors. Moreover, dissection of the embryonic SN at E13.5 ensured the definitive anatomical and developmental origin of our cell line. Another advantage of this modulatable oncogene is that it provided an experimental means for conditional immortalization by shifting the cultivation temperature from the proliferative, 33°C, to the nonproliferative, 38–39°C (Jat et al., 1991; Whitehead et al., 1993). Thus, the conditional immortalization of specific DA neuronal progenitor cells provides a new tool to investigate the regulation of DA neuronal differentiation at the molecular level by specific environmental cues.

### DA phenotypic and morphological differentiations can be monitored and manipulated *in vitro*

In these studies, two different issues were addressed by monitoring the expression of various molecular markers. First of all, can the immortalized SN DA progenitor cell line express known DA neuronal markers? If so, can these DA neuronal markers and morphological changes be differentially manipulated by defined environmental cues, such as temperature, serum, growth factors, and retinoic acid? The immortalized neuronal cell line SN4741, arrested at relatively early developmental stage, expressed high levels of neuronal markers, neurotrophins, and receptors. The SN DA markers, such as $D_2$ autoreceptor and DA-T, except TH, were moderately expressed in the SN4741 cells. In contrast, the expression of a representative DA phenotypic marker, TH, was much lower in the SN4741 cell line grown in 33°C than the mature DA neurons. Thus, we hypothesized that the SN4741 cell line might be at a stage not far from terminal differentiation that is normally characterized by high level TH expression and morphological differentiation. This notion was tested in two different ways. First, the SN4741 cells were cultured under several defined conditions, such as nonpermissive temperature with a minimal serum concentration and/or retinoic acid or at high cell density. Indeed, withdrawal from mitotic cell division produced by both inactivation of the oncogene and the restriction of serum as well as the presence of retinoic acid induced a distinct neuronal morphological differentiation. Increased TH immunostaining (~2.5-fold higher than that at the low-density culture) was achieved at high cell density culture through, as yet, unidentified signaling mechanisms. Second, a culture paradigm similar to the *in vivo* developmental condition was adopted to test whether any non-DA factors are necessary to induce the differentiation of the SN4741 cell line. In confirmation of the early coculture studies (Rousselet et al., 1988; O'Malley et al., 1991), differentiation and survival of SN4741 cell line were improved by coculture with mesencephalic neurons and astrocytes. Although TH immunostaining of the SN4741 cells greatly increased (approximately fivefold to sevenfold) in the cocultures, the cells did not display typical neuron-like morphology. Therefore, in this SN DA progenitor cell line, morphological and phenotypic differentiation can be distinguished as independent events and may be regulated by distinct factors or signaling pathways. This notion may be further tested by transplanting the cell line into embryonic brains (Brustle et al., 1995).

### BDNF provides DA neuroprotection against $MPP^+$-induced neurotoxicity in the SN DA cell line via TrkB

The coexpression of BDNF and TrkB in SN DA neurons and many other neurons of the CNS suggested that BDNF acts in a autocrine and/or paracrine mode (Kokaia et al., 1993; Seroogy et al., 1994). Our SN4741 cell line also expressed both BDNF and TrkB. In the SN4741 cells, a low level of TrkB activation was constitutively observed at >60–70% confluence possibly induced by the secreted BDNF. TrkB activation was significantly increased by exogenous BDNF treatment. The definitive molecular mechanisms of BDNF action via TrkB and regulatory BDNF release in the SN DA neurons have yet to be firmly established, given the paucity of DA neurons and cellular heterogeneity in primary mesencephalic cultures. Employing the SN4741 cell line, the first issue addressed was, did the SN4741 cells show $MPP^+$-induced neurotoxicity and did exogenous BDNF protect against neurotoxicity? Exposure of the SN4741 cells to $MPP^+$ demonstrated DA neuron-specific neurotoxicity. $MPP^+$, taken up via DA-T, is known to inhibit mitochondrial complex I. In fact, the mitochondrial complex I deficits, demonstrated in PD patients using cybrid cell lines, were associated with increased free radical production and apoptotic cell death (Swerdlow et al., 1996). In addition, NO and excitatory glutamate showed similar neurotoxic effects on the SN4741 cell line. The similarities occurred because neuronally derived NO is thought to mediate partially $MPP^+$-induced neurotoxicity in parkinsonian baboons and mice (Hantraye et al., 1996; Przedbroski et al., 1996), and glutamate-induced excitotoxicity is also known to be mediated by NO (Dawson et al., 1993). Therefore, BDNF protected against $MPP^+$, NO, and glutamate-induced neurotoxicity, which may share a common signaling pathway in the SN4741 cell line. In contrast, BDNF was not neuroprotective against dopamine-induced neurotoxicity, which suggests a distinctive mechanism for dopamine-induced versus $MPP^+$-, NO-, and glutamate-induced neurotoxicity in the SN-derived DA neuronal cell line. Dopamine-induced neurotoxicity was shown to be caused by both its toxic free radical products and inhibition of mitochondrial NAD dehydrogenase activity (Duffy et al., 1996; Ben-Shachar et al., 1995). Therefore, in SN DA neurons BDNF appears to exert a rather selective neuroprotection against various neurotoxic insults.

Given the significance of both autocrine and paracrine actions of BDNF in SN, a second issue addressed was: did known neurotoxic insults affect and promote the quantitative release of BDNF from the SN4741 cell line as a self-defensive neuroprotection? To address the issue, we tested five representative classes of known neurotoxic chemicals at sublethal doses, such as mitochondrial electron transport inhibitor ($MPP^+$), free radical generator ($H_2O_2$), NO producer (SNP), excitatory amino acid (glutamate), and proinflammatory cytokine (TNF-$\alpha$), all of which have been suggested to contribute to the SN DA neuronal loss in PD. Of particular interest, glutamate and TNF-$\alpha$ increased the release of BDNF. Glutaminergic stimulation has been known to enhance the level of BDNF mRNA or release of BDNF in the hippocampus, cortex, and cerebellum (Favaron et al., 1993; Wetmore et al., 1994; Figurov et al., 1996). The BDNF upregulation observed may be either a compensatory neuroprotective response against excitotoxicity or involved in the modulation of synaptic function. TNF-$\alpha$ was detected in the SN glial cells of PD patients (Boka et al., 1994) and known to cause either cytotoxic cell death or neuroprotection depending on the overall profile of TNF-$\alpha$ activity (Schulze-Osthoff et al., 1993; Cheng and Mattson, 1994). Thus, in our experimental condition glutamate and TNF-$\alpha$ treatments appear to induce a neuroprotective BDNF release. In contrast, two potential free radical generators, NO and $H_2O_2$, significantly suppressed the BDNF release, but $MPP^+$ did not. Although the significance of this observation is unknown, each neurotoxic stress may affect both the protective BDNF synthesis and release via different signaling mechanisms in DA neurons of SN. Therefore, it is necessary to elucidate the physiological role of the autocrine/paracrine action of BDNF in neurons and glial cells of SN, which may have significant implications in SN DA neuronal survival under various neurotoxic stresses.

In conclusion, our SN DA cell line, SN4741, maintains many of the characteristic features of SN DA neurons and provides not only a new tool to elucidate the molecular mechanisms of BDNF-evoked neuroprotection and neuronal differentiation, but also a model system to investigate the regulatory role of autocrine BDNF release under both normal and stressed conditions. These studies will shed light on the complex molecular mechanisms of DA neuronal survival and death and reveal new molecular targets for PD therapy.

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