Immortalized Dopamine Neurons: A Model to Study Neurotoxicity and Neuroprotection (44439)

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Abstract. **6-Hydroxydopamine (6-OHDA) causes selective degeneration of dopaminergic neurons in the rat brain and has been used to produce an animal model of Parkinsonism. Recently, a clonal line of immortalized dopamine (DA) neurons ($1RB_3AN_{27}$), which expresses varying levels of tyrosine hydroxylase, dopamine transporter, neuron specific enolase, and nestin, was established. These DA neurons reduce behavioral deficits in 6-OHDA–lesioned rats. The relative sensitivity of fetal and adult neurons to potential neurotoxins is not well defined. The availability of immortalized DA neurons provides a unique opportunity to compare the relative neurotoxicity of 6-OHDA in differentiated and undifferentiated DA neurons *in vitro* and identify neuroprotective agents. Our results showed that 6-OHDA treatment for 24 hr decreased the viability of undifferentiated and differentiated immortalized DA neurons *in vitro*, as determined by the MTT assay, and increased the rate of apoptosis in differentiated DA neurons. The differentiated DA neurons (IC_{50} = 33 μ*M*) were about 2-fold more sensitive to 6-OHDA than undifferentiated DA neurons (IC_{50} = 75 μ*M*) in cell culture. Similarly, the differentiated DA neurons were more sensitive to another neurotoxin, 1-methyl-4-phenylpyridinium (MPP^+), which is commonly used to induce Parkinsonism in animal models, than were the undifferentiated DA neurons in culture. Among growth factors tested, only glial cell line–derived neurotrophic factor (GDNF) partially protected differentiated DA neurons against 6-OHDA–induced toxicity. These results suggest that undifferentiated and differentiated immortalized DA neurons can be a useful experimental model to study relative sensitivity to neurotoxins and neuroprotective agents that could have relevance to fetal and adult neurons.**

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The selective degeneration of dopaminergic neurons in the substantia nigra is one of the principal features of the pathogenesis of human Parkinson's disease (PD). The mechanisms of neuronal degeneration in this disease are not fully understood. 6-Hydroxydopamine(6-OHDA) (1, 2) and N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride (MPTP) (3, 4) have been shown to cause selective degeneration of dopamine (DA) neurons and have been used extensively to produce animal models of PD (5–11). We have recently used this model to show that grafting of immortalized DA neurons into striata of 6-OHDA–lesioned rats improves neurological deficits (12, 13). The $1RB_3AN_{27}$ cell line, which produces DA, expresses tyrosine hydroxylase, dopamine transporter, neuron specific enolase, and nestin, was derived from fetal rat mesencephalon by transfection with plasmid vector (pSV_3^{neo}) that carries the LTa gene from SV40 virus (12–15). The availability of immortalized DA neurons provides a unique opportunity to compare the effects of neurotoxins and their modification by chemicals on undifferentiated and differentiated DA neurons.

Certain growth factors, such as glial cell line–derived neurotrophic factor (GDNF) (16–21), platelet-derived growth factor (PDGF) (22), brain-derived neurotrophic fac-

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tor (BDNF) (23), glial cells (24), and vitamin E (25), protect DA neurons *in vitro* and *in vivo* against 6-OHDA toxicity. Similar studies have shown that nerve growth factor (NGF) can protect human neuroblastoma cells from 6-OHDA toxicity (26). Although GDNF has been shown to protect DA neurons against 6-OHDA–induced damage *in vivo* (17–21), it has not be ascertained whether this protection is due to the direct effect of GDNF on DA neurons or an indirect effect mediated through non-neural cells. The demonstration of the protective effect of GDNF on a clonal culture of DA neurons can be useful in the investigation of its direct protection against 6-OHDA-induced toxicity. Therefore, we have investigated the effect of various growth factors such as insulin-like growth factor-1 (IGF-1), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), BDNF, and GDNF on the viability of immortalized undifferentiated and differentiated DA neurons *in vitro* after treatment with 6-OHDA.

We now report the following: 1) 6-OHDA treatment decreases the viability of undifferentiated and differentiated immortalized DA neurons ($1RB_3AN_{27}$) with the differentiated cells being more sensitive than the undifferentiated ones; 2) differentiated DA neurons undergo apoptosis at a higher rate than undifferentiated DA neurons in the presence of 6-OHDA; 3) MPP^+ significantly decreases the viability of only differentiated DA neurons; and 4) GDNF partially protects only differentiated immortalized DA neurons against 6-OHDA-induced damage.

Materials and Methods

Cell Culture. The clonal cell line of immortalized DA neurons ($1RB_3AN_{27}$), established in our laboratory, was used (14). Cells were plated on 30-mm plastic Petri dishes at 25,000 viable cells per dish (3500 cells/cm^2). Dishes were precoated with a serum-free MCDB-153 medium (Sigma, St. Louis, MO) containing specialized substrate collagen (30 mg/l, Celtrix, Santa Clara, CA), fibronectin (10 mg/l, Collaborative Biomedical Products, Bedford, MA), and bovine serum albumin (100 mg/l, Sigma) (13). Cells were grown in RPMI-1640 media (Gibco, Grand Island, NY) containing 10% fetal calf serum (Gibco), penicillin G (100 μg/ml, Gibco), and streptomycin (100 units/ml, Gibco), and were maintained at 37°C in a humidified atmosphere of 5% CO_2. Mycoplasma-free cultures were used for all experiments.

Induction of Differentiation. Dibutyryl adenosine 3′,5′-cyclic monophosphate (dbcAMP, Sigma, dissolved in H_2O) at 2 m*M* and dehydroepiandrosterone (DHEA, Sigma, dissolved in ethyl alcohol) at 60 μg/ml were added immediately one after another at 24 hr after plating to induce differentiation (Prasad KN *et al.*, unpublished observation). After 48 hr of treatment, differentiated DA neurons were used for experiments. Differentiation was determined by formation of neurites, enlargement of cell bodies, and increases in tyrosine hydroxylase activity (12) and mRNA levels for tyrosine hydroxylase and dopamine transporter (13). Photographs of this differentiation process have been published in several scientific journals and thus are not presented in this manuscript (12–15).

Treatment of Undifferentiated and Differentiated DA Neurons with 6-OHDA and MPP^+. To test the toxicity of 6-OHDA (RBI, Natick, MA), various concentrations of this neurotoxin were added to cultures containing 25,000 differentiated (0–500 μ*M*) or undifferentiated (0–2 m*M*) DA neurons. The higher 6-OHDA concentrations were used on the undifferentiated DA neurons because preliminary data had shown that these DA neurons had a higher tolerance for 6-OHDA. To test the specificity of the 6-OHDA effect, the active metabolite of MPTP (MPP^+) was added in the same manner as 6-OHDA (0–1 m*M*, RBI). There is evidence that 6-OHDA acts through a superoxide radical as an oxidative intermediate (27, 28). To lessen the degradation of 6-OHDA and keep conditions constant, both 6-OHDA and MPP^+ were dissolved in distilled H_2O containing 100 μg/ml ascorbic acid. All cultures used in this study contained a final concentration of 20 μg/ml ascorbic acid, including the solvent-treated cultures.

Undifferentiated DA neurons were exposed either to 6-OHDA or MPP^+ 24 hr after plating. However, since differentiation takes 72 hr, differentiated DA neurons were exposed either to 6-OHDA or MPP^+ 72 hr after plating. Cell viability was measured 24 hr after treatment with toxins using an MTT assay technique.

To better determine the pharmacological effects of 6-OHDA on immortalized DA neurons, additional $1RB_3AN_{27}$ cultures were exposed to 6-OHDA (0–750 μ*M*) for only 2 hr, and then fresh growth medium containing 20 μg/ml of ascorbic acid was added. Cell survival was then measured 22 hr later by the MTT assay. Toxicity was determined by comparing untreated cultures to treated cultures and then applying one-way analysis of variance (ANOVA) for independent samples using the INSTAT statistical program (GraphPad, San Diego, CA).

Treatment with Growth Factors. To test if neurotrophic agents, known to support fetal DA neurons in primary cell cultures, can block the toxic effects of 6-OHDA on immortalized DA neurons, these agents were added separately to cultures 24 hr before 6-OHDA treatment. The following growth factors at various concentrations were added separately to cultures containing 25,000 differentiated or undifferentiated cells: GDNF ((16), 0–100 ng/ml, Synergen, Boulder, CO), IGF-I ((29), 300–1500 ng/ml, Cephalon, West Chester, PA), bFGF ((29), 4–150 ng/ml, SCIOS, Mountain View, CA), EGF ((30), 40–2000 ng/ml, PeproTech, Rocky Hill, NJ), and BDNF ((31), 400–1000 ng/ml, PeproTech). Since it has been shown that IGF-1 and bFGF have an additive effect on DA neuron survival in primary cultures (29), additional cultures of immortalized DA neurons were exposed to two combinations of IGF-1 and bFGF (300/30 ng/ml and 1,500/150 ng/ml) 24 hr prior to 6-OHDA treatment.

MTT Assay. A water-soluble tetrazolium salt, 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma), was used to determine the number of viable cells by a previously described method (32). The assay was dependent on the cellular reduction of MTT by the mitochondrial dehydrogenase of viable cells, to a blue formazan product, which can be measured by a spectrophotometer. Briefly, MTT was dissolved in RPMI-1640 without phenol red (Sigma) at a concentration of 5 mg/ml. This stock solution was diluted directly into the media for the immortalized DA neurons to give a final concentration of 0.2 mg/ml. During a 4-hr incubation at 37°C, a portion of the MTT was converted to an insoluble purple formazan by cleavage of the tetrazolium ring by dehydrogenase enzymes. The medium was removed, and the formazan product was dissolved with 0.4 ml DMSO. Absorbance was then determined at 540 nm.

Determination of Apoptosis. Cells were plated in 8-chambered glass slides at the same density as in the experiments measured by MTT assay (i.e., 3500 viable cells/cm^2). After 24 hr the undifferentiated cells were treated with 100 μM 6-OHDA or distilled H_2O. For the differentiated cells, differentiation took place over 72 hr, and cultures were treated with 50 μM 6-OHDA or distilled H_2O. All cultures contained a final concentration of 20 μg/ml ascorbic acid. After a 6-hr exposure to 6-OHDA, cells were fixed with 1% paraformaldehyde followed by 70% ethanol in 50 mM glycine buffer (pH 2) for 10 min at –20°C. Nuclei were made fluorescent by incubation with the DNA intercalating dye, Hoechst 33258 (Sigma) at 8 μg/ml for 5 min at 25°C. Fluorescence was observed under a fluorescence microscope (Zeiss), with excitation at 365 ± 20 nm, emission at ≥420 nm. Apoptotic DA neurons were defined as those with fragmented nuclei containing one or multiple lobes of condensed chromatin. To minimize the use of space, we have chosen not to publish a photo of an apoptotic nucleus; we have already published such images extensively (29, 33, 34). Apoptotic TH^+ neurons were counted in cultures treated with 6-OHDA and in control cultures and compared by Student's t test using the INSTAT statistical program. At least 10 fields/well were examined containing at least 50 cells/field, yielding $n \geq 500$ cells examined/well.

Results

Effect of 6-OHDA on Viability. The linear relationship between the number of viable immortalized DA neurons per plate and absorbance of the formazan in DMSO holds very well in the 5,000–25,000 cell/plate range in which all the experiments were conducted. The standard curve for differentiated immortalized DA neurons is shown in Figure 1. For every dose-response curve, a similar standard curve was generated exactly matching the incubation and differentiation conditions. The line fit to the data shown in Figure 1 was used to generate the dose-response curve in Figure 2A.

The effect of 6-OHDA on the viability of differentiated and undifferentiated DA neurons was investigated using the MTT assay technique. Results showed that 6-OHDA treatment for 24 hr or 2 hr decreased the viability of differentiated DA neurons (Figs. 2A and 2B) and that 6-OHDA treatment for 24 hr decreased the viability of undifferentiated DA neurons (Fig. 2C); however, the differentiated cells were more sensitive than undifferentiated cells. The IC_{50} values for a 24-hr exposure of differentiated and undifferentiated DA neurons to 6-OHDA were 33 μM and 75 μM, respectively, and the IC_{50} value for a 2-hr exposure of differentiated DA neurons to 6-OHDA was 50 μM. Addition of the solvent alone (final concentration of ascorbic acid 20 μg/ml) had no effect on the survival of either differentiated or undifferentiated DA neurons when compared to untreated controls.

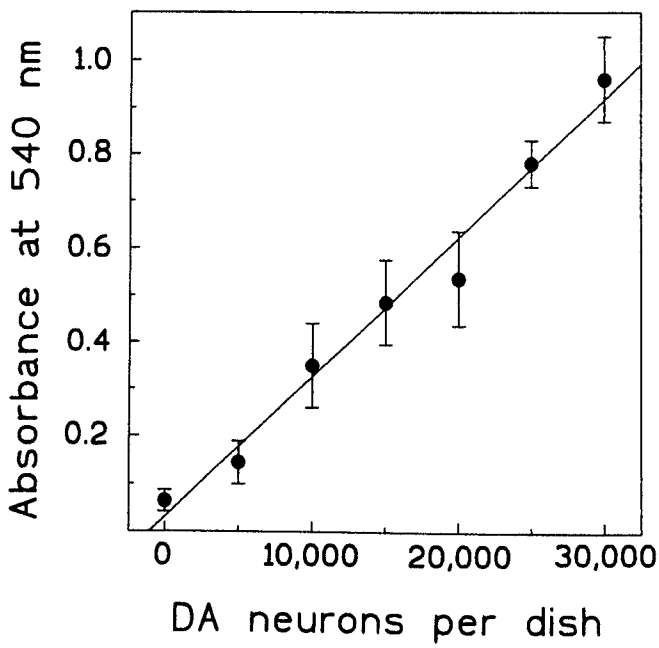

Figure 1. Standard curve of differentiated immortalized DA neurons vs. response of MTT assay. Immortalized DA neurons ($1RB_3AN_{27}$ cells) were plated at concentrations ranging from 5000 to 30,000 viable cells/30-mm dish and differentiated. After differentiation, 0.2 mg/ml MTT were added to the medium for 4 hr at 37°C, then the medium was removed and the formazon product dissolved in DMSO. Absorbance was measured at 540 nm and plotted as a function of cell number. The data were fit by linear regression resulting in the line: Cell # = 33,800 A_{540}–1,000, with a correlation coefficient of r = 0.99. Each point represents the mean ± SEM (n = 4).

Effect of 6-OHDA on Apoptosis. The effect of 6-OHDA on the rate of apoptosis in differentiated and undifferentiated DA neurons was examined by adding 50 μM 6-OHDA to differentiated DA neurons and 100 μM 6-OHDA to undifferentiated DA neurons and measuring apoptosis, as determined by nuclear fragmentation, 6 hr later. The overall rate of apoptosis in all of the cultures was fairly low with no culture having more than 10% of its cells apoptotic at any time. However, 6-OHDA treatment did significantly increase the rate of apoptosis in differentiated DA neurons from 2.5% apoptotic to 4.5% apoptotic (Fig. 3). Although undifferentiated DA neurons did have a moderate

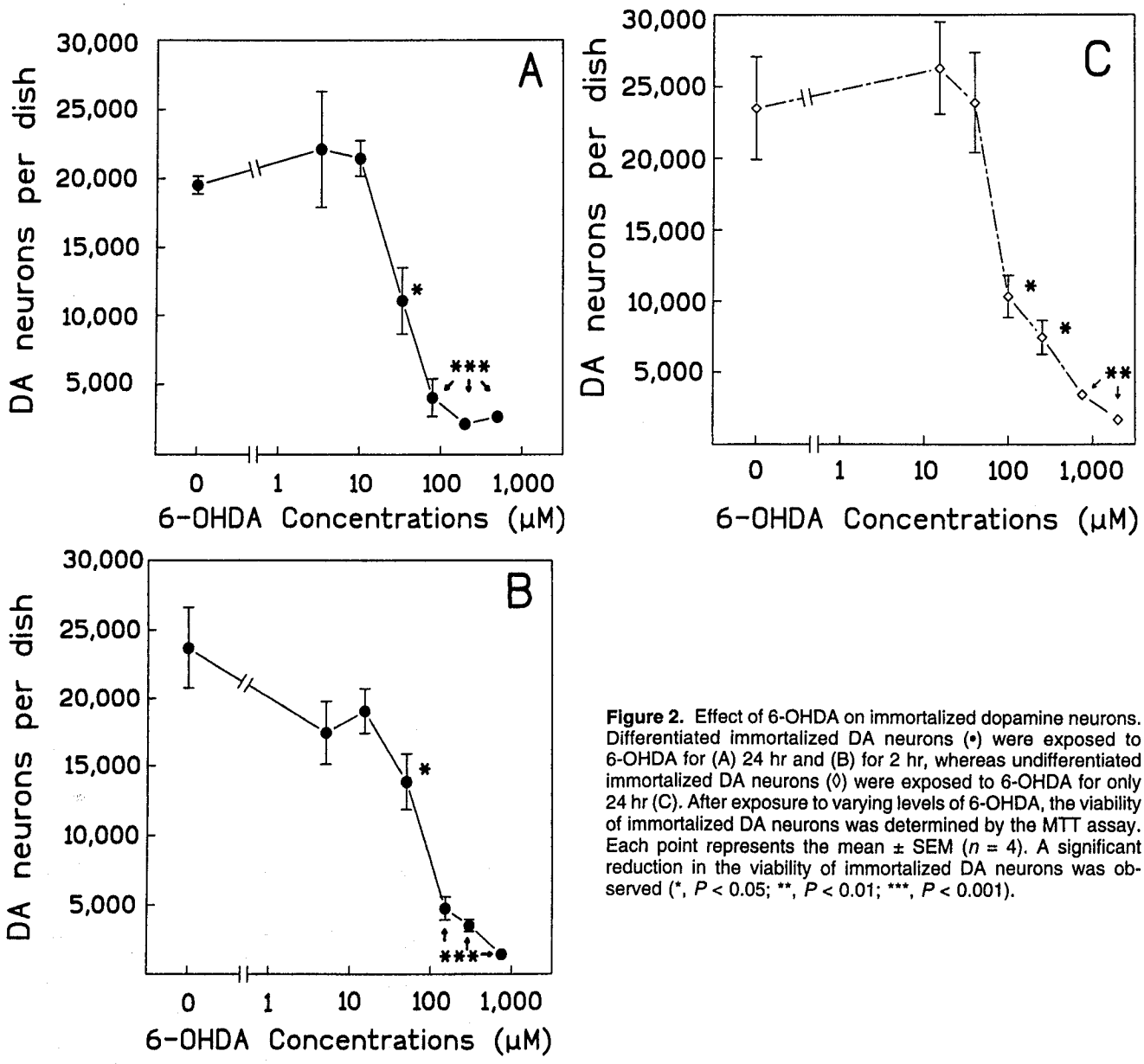

Figure 2. Effect of 6-OHDA on immortalized dopamine neurons. Differentiated immortalized DA neurons (•) were exposed to 6-OHDA for (A) 24 hr and (B) for 2 hr, whereas undifferentiated immortalized DA neurons (◊) were exposed to 6-OHDA for only 24 hr (C). After exposure to varying levels of 6-OHDA, the viability of immortalized DA neurons was determined by the MTT assay. Each point represents the mean ± SEM (n = 4). A significant reduction in the viability of immortalized DA neurons was observed (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

increase in apoptosis when exposed to 6-OHDA (from 1.6% to 2.2%), the difference was not statistically significant.

Effect of MPP^+ on Viability. The effect of another neurotoxin, MPP^+, which has also been used to produce primate models of PD (8–11), was investigated in the same manner as described for 6-OHDA. Results showed that differentiated DA neurons were more sensitive to MPP^+ than undifferentiated ones (Figs. 4A and 4B). There was no statistically significant decrease in undifferentiated DA neuron survival even at a concentration of 1 mM, MPP^+. Doses greater than 1 mM MPP^+ were not examined since these concentrations would not be relevant to *in vivo* conditions.

Effect of Neurotrophic Factors on 6-OHDA–Induced Toxicity. The effect of growth factors, such as GDNF, BDNF, EGF, bFGF, and IGF-1 on the viability of 6-OHDA–treated differentiated and undifferentiated DA neurons, was investigated. Results showed that treatment of differentiated DA neurons with GDNF for 24 hr before addition of 50 μM 6-OHDA increased the viability of 6-OHDA–treated cells. However, when undifferen-tiated DA neurons were treated similarly with GDNF and 100 μM 6-OHDA, GDNF failed to provide any protection (Fig. 5). The optimal protection was achieved at a GDNF concentration of ≥25 ng/ml. Treatment with other growth factors such as IGF-1 (300–1500 ng/ml), bFGF (4–150 ng/ml), BDNF (400–1000 ng/ml), EGF (40–2000 ng/ml), and two combinations of IGF-1/bFGF (300/30 ng/ml and 1,500/150 ng/ml) were ineffective in protecting 6-OHDA–treated differentiated DA neurons (data not shown).

Discussion

This study shows that differentiated immortalized DA neurons are more sensitive to both 6-OHDA and MPP^+ than undifferentiated immortalized DA neurons in culture as

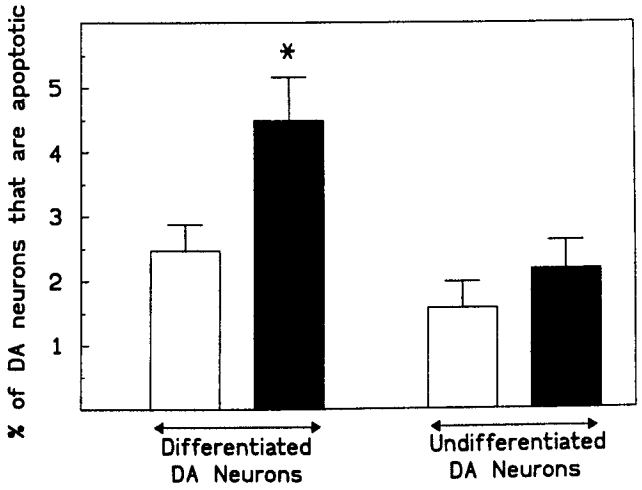

Figure 3. Effect of 6-OHDA treatment on the rate of apoptosis in immortalized DA neurons. Immortalized DA neurons were plated in eight chambered plastic slides (3,500/cm^2) with half the slides being differentiated before the addition of 6-OHDA (50 μ*M* to differentiated DA neurons and 100 μ*M* to undifferentiated DA neurons). After 6 hr of 6-OHDA treatment, the rate of apoptosis was determined by the nuclear fragmentation technique. Clear bars are controls, and black bars are 6-OHDA–exposed cultures. Each bar represents the mean ± SEM ($n = 5$). A significant increase in apoptosis occurred in differentiated DA neurons exposed to 6-OHDA (*, $P < 0.05$).

measured by viability and rate of apoptosis. This suggests that fetal dividing nerve cells may be relatively resistant to certain neurotoxins such as 6-OHDA and MPP$^+$. The exact reasons for the differential effect of these neurotoxins on differentiated and undifferentiated immortalized DA neurons are not well understood, with one of the possible mechanisms of action of 6-OHDA and MPP$^+$ involving free radicals. However, we have proposed that epigenetic changes involving mitochondria, membrane structures, and protein modification in differentiated neurons are primarily targets for the action of neurotoxins including free radicals, which lead to cell death (35). In contrast, genetic changes in undifferentiated and dividing DA neurons may be the primary targets for cell death (35). These genetic changes could potentially be repaired. On the other hand, epigenetic components of differentiated DA neurons do not contain repair systems; therefore, they cannot recover from damage. These differences in their biology may in part account for the higher sensitivity of differentiated neurons to neurotoxins. The 6-OHDA–induced toxicity has also been observed in primary cultures of fetal rat mesencephalic cells containing DA neurons (36), murine undifferentiated neuroblastoma cells (37, 38), bovine adrenal chromaffin cells (28), and rat brain synaptosomes (39) *in vitro*. It is also known that 6-OHDA induces apoptosis in PC12 cells (40–42) and thymocytes (43). Similarly, the neurotoxicity of MPP$^+$ has been observed *in vitro* (36) and *in vivo* (8–11). Thus, our results suggest that for fetal and adult neurons, undifferentiated and differentiated immortalized DA neurons can be used as models to study the mechanisms of action of neurotoxins that are presumed to have selective effects on DA neurons.

The present study shows that GDNF was effective in partially protecting only differentiated immortalized DA neurons against 6-OHDA–induced toxicity; however, bFGF was ineffective. Others have reported that bFGF protects DA neurons from 6-OHDA toxicity in primary cultures (44). Since it has been shown that at least part of bFGF's neurotrophic effect is mediated through glial cells (29), it is not surprising that bFGF did not have any protective effect on clonal nerve cells in culture.

The mechanisms by which GDNF protects differentiated DA neurons are unknown. It is possible that GDNF promotes repair of 6-OHDA–induced damage, upregulates antioxidant enzyme systems, or reduces the uptake of 6-OHDA. Since differentiated DA neurons have increased levels of tyrosine hydroxylase and dopamine transporter, it is possible that differentiation is also increasing the expression of GDNF receptors. Our results also suggest the possibility that the component of 6-OHDA–induced damage not reduced by GDNF may be due to free radicals that are generated during auto-oxidation of 6-OHDA (27, 28). This suggestion is supported by the fact that antioxidant vitamins such as vitamin E, protect from 6-OHDA–induced neurotoxicity *in vivo* (25). Thus 6-OHDA–induced neurotoxicity involves at least two mechanisms one of which is influenced by certain growth factors and the other by antioxidant vitamins.

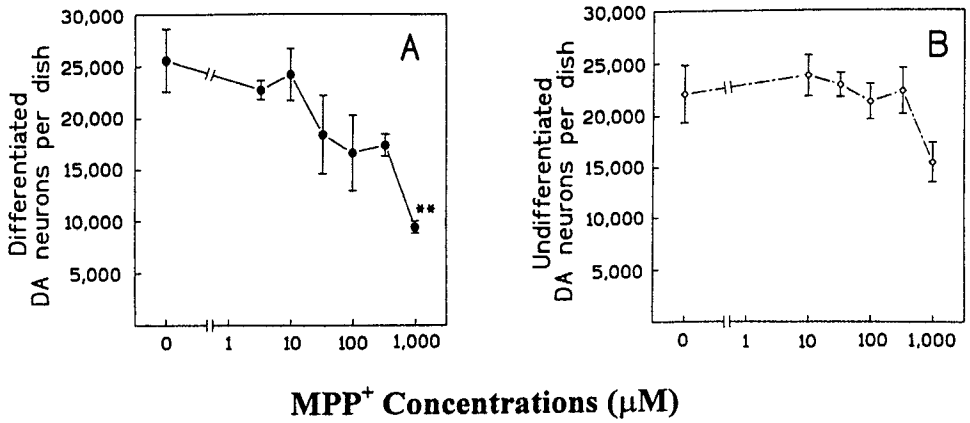

Figure 4. Effect of MPP$^+$ on immortalized DA neurons. (A) Differentiated (•) or (B) undifferentiated (◊) DA neurons were plated in tissue culture dishes (25,000/30 mm), and MPP$^+$ at various concentrations was added. After 24 hr of treatment, the viability of the cells was determined by MTT assay. Each point represents the mean ± SEM ($n = 4$). A significant reduction in viability was observed in differentiated DA cells at 1 m*M* MPP$^+$ (**, $P < 0.01$). However, no significant reduction in the number of undifferentiated neurons was achieved.

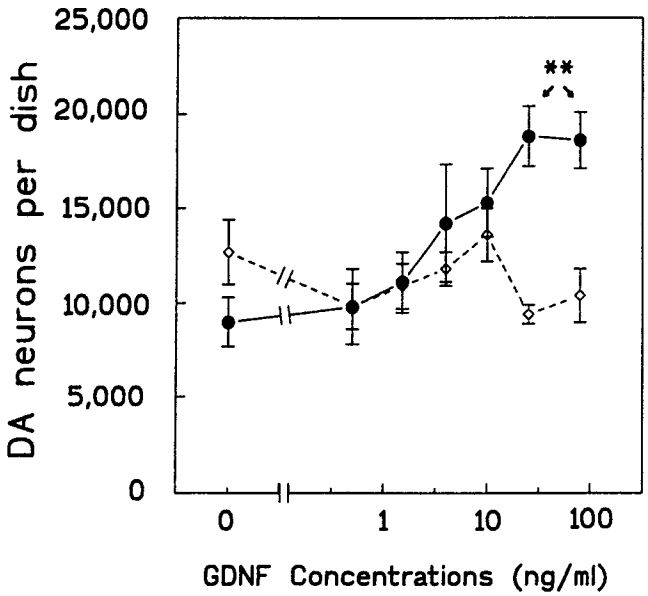

Figure 5. Effects of GDNF on 6-OHDA–induced toxicity in differentiated and undifferentiated immortalized DA neurons. Differentiated (•) or undifferentiated (◊) immortalized DA neurons were plated in tissue culture dishes (25,000/30 mm), and various concentrations of GDNF were added 24 hr later. After exposure to GDNF for 24 hr, differentiated DA neurons were then treated with 50 μ*M* 6-OHDA and undifferentiated DA neurons with 100 μ*M* 6-OHDA. After exposure to 6-OHDA for 24 hr, viability was determined by MTT assay. Each point represents the mean ± SEM ($n = 4$). A significant increase in the number of surviving differentiated cells was observed in GDNF-treated cultures at concentrations of 25 ng/ml and 80 ng/ml (**, $P < 0.01$) when compared to untreated control.

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