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A Synergistic Neurotrophic Response to *l*-Dihydroxyphenylalanine and Nerve Growth Factor

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ABSTRACT

The catecholamine precursor *l*-dihydroxyphenylalanine (L-DOPA) is the primary therapeutic intervention for Parkinson's disease. Although short-term exposure (30 min) potentiates dopamine (DA) release by elevating quantal size, longer term exposure to L-DOPA (48 hr) promotes neurite outgrowth from midbrain DA neurons in culture. To characterize long term effects of L-DOPA, we used a pheochromocytoma (PC12) line that extends neurites on exposure to nerve growth factor (NGF). L-DOPA potentiated the outgrowth of processes elicited by NGF. This response did not require conversion of L-DOPA to DA, was not caused by agonist effects at DA receptors, and was not blocked by the tyrosine kinase inhibitor genistein. However, similar results were found after exposure to *l*-*n*-acetylcysteine or apomorphine, a DA receptor agonist that produces a quinone metabolite, and seemed to correlate with glutathione synthesis. Long-term process elaboration was blocked by L-buthionine sulfoximine, consistent with mediation by an antioxidant mechanism. L-DOPA potentiation of NGF response was important functionally as seen by increased quantal neurotransmitter release from the L-DOPA/NGF-treated neurite varicosities, which displayed both 2-fold greater quantal size and frequency of quantal release. These results demonstrate potentiation by L-DOPA of morphological and physiological responses to neurotrophic factors as well as synergistic induction of antioxidant pathways. Together with effects on transmitter synthesis, these properties seem to provide a basis for the compound's long term presynaptic potentiation of DA release and therapeutic actions.

The catecholamine precursor L-DOPA elicits either neurotoxic or neurotrophic responses depending on experimental conditions. Toxic effects have been reported in neuroblastoma lines and catecholamine neurons cultured in the absence of astrocytes; this seems to be caused by a promotion of oxygen radicals, because the toxicity is prevented by the antioxidant ascorbic acid (Mena *et al.*, 1993; Pardo *et al.*, 1993; Pardo *et al.*, 1995), overexpression of superoxide dismutase (Mena *et al.*, 1997c), and the monoamine oxidase inhibitor deprenyl (Mena *et al.*, 1992), which inhibits production of hydrogen peroxide (Cohen and Spina, 1989). Moreover, L-DOPA toxicity is correlated with high extracellular levels of quinone oxyradical L-DOPA derivatives (Graham, 1978; Mena *et al.*, 1992; Basma *et al.*, 1995).

Paradoxically, lower L-DOPA exposures (25–200 μM) are selectively neurotrophic for DA neurons in ventral midbrain astrocyte cocultures, promoting cell survival (Mytilineou *et al.*, 1993; Mena *et al.*, 1997b) and neurite outgrowth (Mena *et al.*, 1997b). The trophic effects may be caused by factors synthesized in astrocytes that are upregulated by L-DOPA exposure (Han *et al.*, 1996; Mena *et al.*, 1997b). Indeed, astrocyte-conditioned medium alone protects against L-DOPA neurotoxicity in glial-free cultures of midbrain DA neurons (Mena *et al.*, 1997a), as would be expected if soluble neuroprotective glial-derived factors were released by astrocytes.

Recently, it has become evident that neurotrophic factors affect neurotransmitter release as well as cell survival and development. The striking ability of L-DOPA to induce neurite outgrowth in culture may provide a basis for long-lasting changes in transmitter release, and the often observed delay in full therapeutic benefit after L-DOPA treatment for Parkinsonian disorders. Although target-derived growth factors (including glial-derived neurotrophic factor) increase neurite arborization of midbrain DA neurons (Lin *et al.*, 1993; Pothos *et al.*, 1998), there is at present no method to trigger novel initiation of central DA neurite outgrowth. We therefore examined PC12 cells, a DA-secreting pheochromocytoma cell line that produces neurites on exposure to NGF (Greene and Tischler, 1976). We find that L-DOPA potentiates neurotrophin-elicited outgrowth and that this response results in

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ABBREVIATIONS: L-DOPA, *l*-dihydroxyphenylalanine; BSO, L-buthionine sulfoximine; DA, dopamine; DCF, 2,7-dichlorofluorescin diacetate; GSH, glutathione (reduced form); LNAC, *l*-N-acetylcysteine; NGF, nerve growth factor; ANOVA, analysis of variance.

elevated quantal neurotransmission from the neurite varicosities. An emerging hypothesis from these studies as well as studies in other systems (Sundaresan *et al.*, 1995) is that oxidative metabolism strongly influences the presynaptic neurosecretory apparatus via a synergistic response with neurotrophic factors.

Materials and Methods

Cell culture. PC12 cells were obtained from Dr. Lloyd Greene (Department of Pathology Columbia University, New York, NY) and cultured as described (Greene and Tischler, 1976) using RPMI 1640 medium supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum (JRH Biosciences, Lenexa, KS). The cells were plated onto plastic dishes or multiwell plates (Costar, Cambridge, MA) coated with rat tail collagen (Vitrogen 100; Collagen Biomedical, Palo Alto, CA) or glass coverslips (Carolina Biological Supply #2, Burlington, NC) coated overnight with 40 μg/ml poly-*d*-Lys (molecular mass, 70–150 kDa; Sigma, St. Louis, MO) and then recoated with 10 μg/ml laminin for 2 hr (Collaborative Biomedical, Bedford, MA). Cell density platings were as follows: for fluorescent observations, processes measurements, and electrophysiology, 40,000 cells per ml were plated on 1-cm^2 glass coverslips in a 50-mm diameter Petri dish (total medium volume, 2.5 ml); for cell counts, 50,000 cells were plated per well in 24-well plates (total medium volume, 1 ml); for GSH and protein measurements 400,000 cells were plated in 6-well plates (total medium volume, 4 ml). NGF (human recombinant) was donated by Genentech (South San Francisco, CA) and used at a concentration of 50 ng/ml. Sulpiride was from Research Biochemicals (Natick, MA); other compounds were obtained from Sigma except where noted.

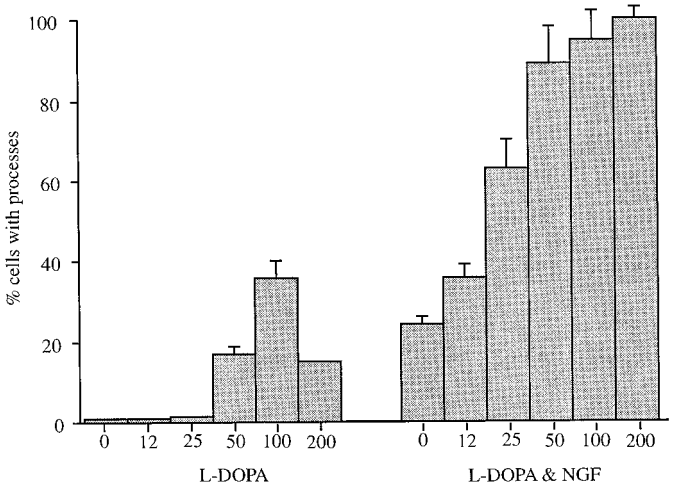

Fig. 1. Effect of L-DOPA and NGF on induction of neurites. PC12 cultures at 2 days after plating were treated for 24 hr with L-DOPA with or without NGF (50 ng/ml). The percentage of cells that exhibited neurites are reported as mean ± standard error (n = 10 cultures). L-DOPA increased neurite expression both by itself (*leftmost bars*) and with NGF (*rightmost bars*; $p < 0.001$ by ANOVA for both cases).

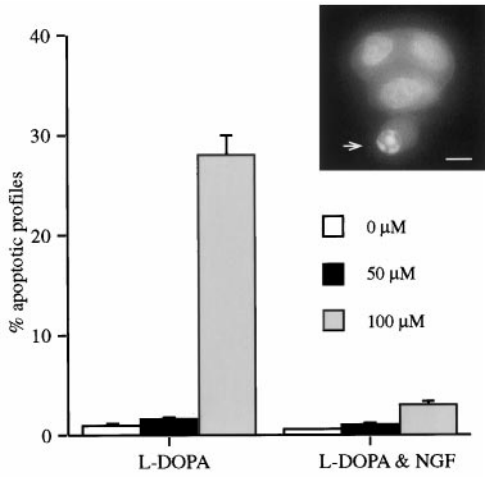

Fig. 2. Effect of L-DOPA and NGF on apoptosis. To test induction of apoptosis by L-DOPA, cultures at 2 days after lating were treated for 24 hr with L-DOPA (0, 50 and 100 μM) with and without NGF (50 ng/ml). The number of apoptotic cells labeled with Hoechst 33342 are indicated. Results are expressed as mean ± standard error (n = 8 cultures). NGF protected the cells from L-DOPA-induced apoptosis ($p < 0.001$, ANOVA). *Inset*, four stained nuclei; *arrow*, apoptotic profile. *Scale bar*, 5 μm.

Cell measurements. We adapted semiquantitative methods for estimating the number and elaboration of processes in culture (Denis-Donini *et al.*, 1983). In each culture, 8–10 consecutive fields (magnification, 100× or 200×) were counted under phase optics. Results are expressed as mean ± standard error values for two to four independent experiments. Each data point corresponds to four or more cultures. Images were produced using an inverted microscope (Axiovert 135 TV; Carl Zeiss, Thornburg, NY), a digital camera (Star I CCD; Photometrics, Tucson, AZ) and National Institutes of Health Image software.

Electrochemical techniques. Amperometric detection of DA secretion in real time was as described (Pothos *et al.*, 1996; Pothos *et*

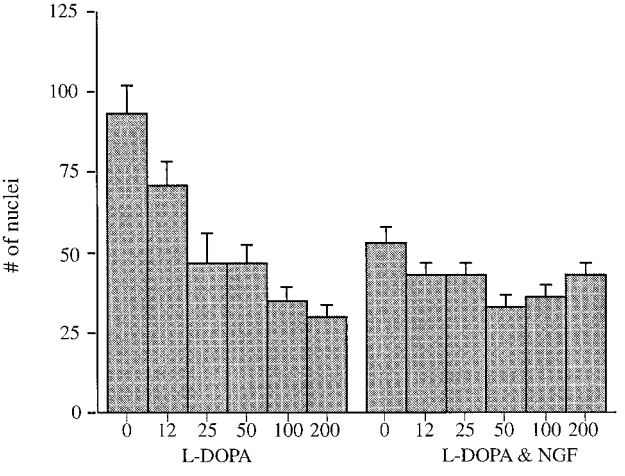

Fig. 3. Effect of L-DOPA and NGF on cell number. PC12 cultures at 2–3 days after plating were treated with L-DOPA (0, 12, 25, 50, 100, and 200 μM) for 24 hr with and without NGF (50 ng/ml). The number of surviving cells (as represented by the number of intact nuclei) are indicated. Results are expressed as mean ± standard error (n = 10–16 cultures). L-DOPA decreased the cell number both with and without NGF ($p <$ 0.001, ANOVA); however, this effect was reduced in the presence of NGF. Note that the total numbers of cells was reduced by NGF alone, as it induced differentiation and inhibited cell division.

TABLE 1

Effects of L-DOPA and NGF on intracellular DCF label

Five days after plating, cultures were exposed to 0, 50, or 200 μM L-DOPA with or without NGF (50 ng/ml) for 4 days. The cultures were then examined for cells labeled by DCF. Data are mean ± standard error (n = 6; at least 2000 cells counted per condition).

	% Labeled cells	
	no NGF	+ NGF
No L-DOPA	0.51 ± 0.37	0.48 ± 0.35
50 μM L-DOPA	3.83 ± 0.66*	0.51 ± 0.46**
200 μM L-DOPA	6.20 ± 0.82*	0.96 ± 0.36**

* $p < 0.001$ versus no L-DOPA.

** $p < 0.001$ for cultures treated with NGF/L-DOPA versus the same level of L-DOPA without NGF.

al., 1998) except that electrodes were placed on processes rather than cell bodies. The cultures were examined 1–2 weeks after plating. Data was analyzed using a locally written program in the Superscope II environment (GW Instruments, Sommerville, MA). The average background current in the vicinity of the spikes was subtracted from the signal and spikes were identified if their amplitude was 4.5 times greater than the root-mean-square background current. The number of molecules (quantal size) oxidized at the electrode face was determined by the relation $N = Q/n\mathrm{F}$, where Q is the charge of the spike, n is the number of electrons transferred [shown to be two for catecholamines when used in a similar experimental configuration (Ciolkowski *et al.*, 1994)], N is the number of moles and F is Faraday's constant (96,485 coulombs per equivalent). Additional parameters that were measured for each quantal event were: i_{max}, the maximum amplitude; $t_{½}$ the width at half i_{max}; *width*, defined as duration between the start of the upward slope and the point at

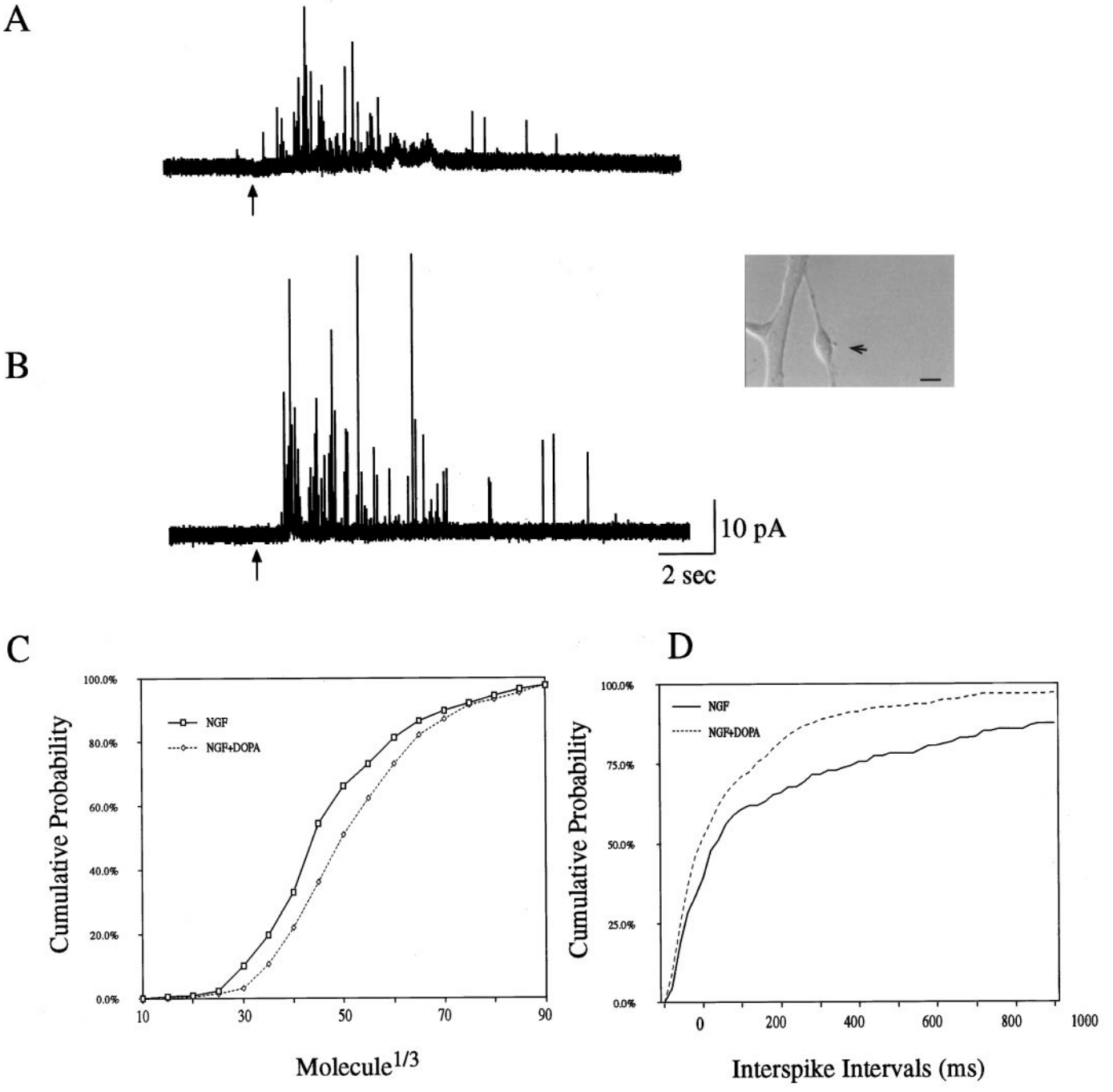

Fig. 4. Effect of L-DOPA on quantal release. A, An amperometric recording of quantal release from a representative PC12 varicosity 1 week after NGF exposure (50 ng/ml). Secretion was stimulated by 80 mM K^+ administered for 3 sec (*arrow*), evoking multiple quantal events. *Inset*, example of a representative PC12 varicosity. *Scale bar*; 2 μm. B, An amperometric recording from a representative PC12 varicosity 1 week after exposure to both NGF and 50 μM L-DOPA. C, Cumulative distribution of the cubed root of the quantal sizes of control and L-DOPA-treated cultures as reported in Table 1. The cubed root of the number of DA molecules are displayed. The population of quantal sizes in the L-DOPA and NGF-treated group is shifted to higher values than NGF alone ($p < 0.0001$; Komolgorov-Smirnov test, KS-Z = 2.2258). D, The cumulative distribution of intervals between quantal events reported in Table 1. Cells treated with L-DOPA and NGF have a mean frequency of release ~ 2-fold higher than NGF alone (Table 1) and the population as a whole is shifted to shorter interspike intervals ($p < 0.005$, KS-Z = 2.0442).

which the decay reaches baseline; and the interspike interval, a parameter that indicates the release frequency.

Vital nuclear staining. The membrane-permeant bisbenzimide dye Hoechst 333342 (Molecular Probes, Eugene, OR) was used to stain nuclei to identify apoptotic nuclei. A concentrated stock (2 mg/ml) was prepared in water and sterilized by filtration. The stock was diluted 1:50 in sterile phosphate-buffered saline (22.8 mM monobasic sodium phosphate, 76.8 mM dibasic sodium phosphate, 154 mM sodium chloride, pH 7.3) 10 μl was added directly to 100 μl of physiological medium (see below), yielding a final concentration of 4 μg/ml.

Total cell counts. For counts of living cells, plasma membranes were lysed as described (Ferrari *et al.*, 1995) and intact nuclei with evident limiting membranes counted (10–16 cultures per condition).

GSH measurements. GSH levels were measured by the method of Tietze (1969). Briefly, 2×10^6 cells were washed twice with phosphate-buffered saline, lysed with 3% perchloric acid for 15 min at 4¼, and centrifuged; supernatants were neutralized with 9 volumes of 0.1 M NaH_2PO_4, 5 mM EDTA, pH 7.5. GSH content was measured by the addition of 5,5′-dithio-bis-(2-nitrobenzoic acid) and the reaction was monitored at 412 nm. GSH is expressed as a function of total protein of the cell extract. In concordance with previous studies in PC12 cells (Ferrari *et al.*, 1995; Pan and Perez-Polo, 1996), no oxidized glutathione was detectable in the cells by the method of Griffith (1980). Protein was measured in the pellet by the method of Bradford (1976) using bovine serum albumin as the standard.

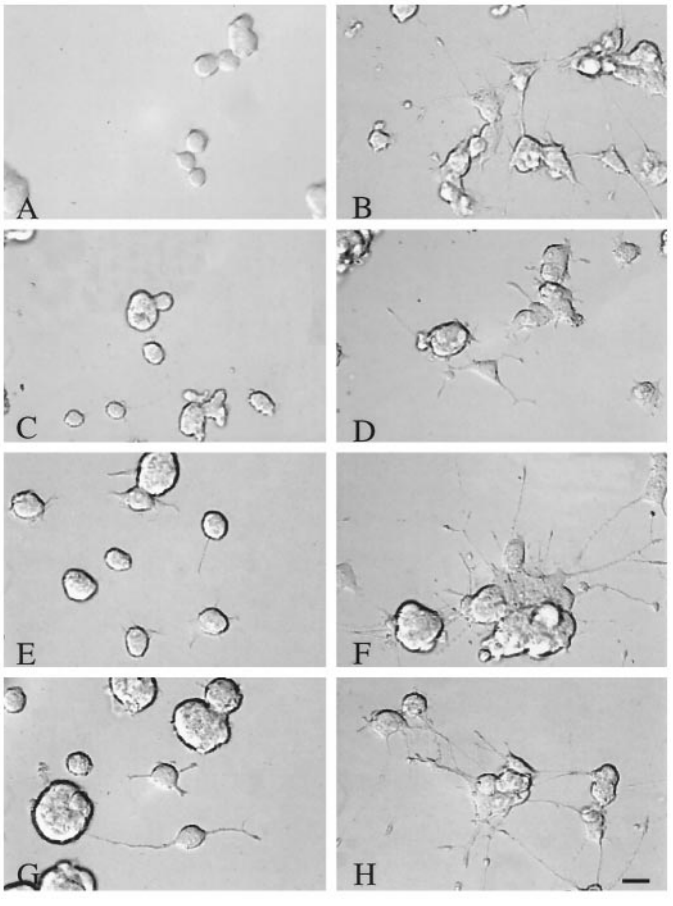

Fig. 5. Effects of carbidopa on L-DOPA and NGF-induced process outgrowth. PC12 cultures at 2 days after plating were treated for 24 hr with vehicle (A), 50 μM L-DOPA (B), 25 μM carbidopa (*CBD*) (C), L-DOPA with carbidopa (D), vehicle and NGF (50 ng/ml) (E), NGF with L-DOPA (F), NGF with carbidopa (G), and NGF with L-DOPA and carbidopa (H). Carbidopa did not block process outgrowth evoked by either L-DOPA or NGF. *Scale bar*, 10 μm.

Measurement of quinones. Quinone formation, which can be used to observe the rate of L-DOPA autoxidation, was evaluated according to the spectrometric measurement of the absorbance at 490 nm in the culture media (Mena *et al.*, 1992; Mena *et al.*, 1993).

DCF label in culture. Aliquots of 10 mM DCF were prepared in dimethylsulfoxide and stored at −85°. Immediately before use, an aliquot was diluted to 1 mM in physiological medium and the cells were labeled with DCF (50 μM, 30 min) in physiological medium. The cultures were then washed three times in Hanks' balanced saline solution containing 10 mM HEPES buffer and 10 mM glucose.

Statistical analysis. The results were statistically evaluated for significance by ANOVA for multiple groups followed by Tukey-Kramer *post hoc* testing for pairs where appropriate. Differences were considered significant at $p < 0.05$. For comparison of quantal

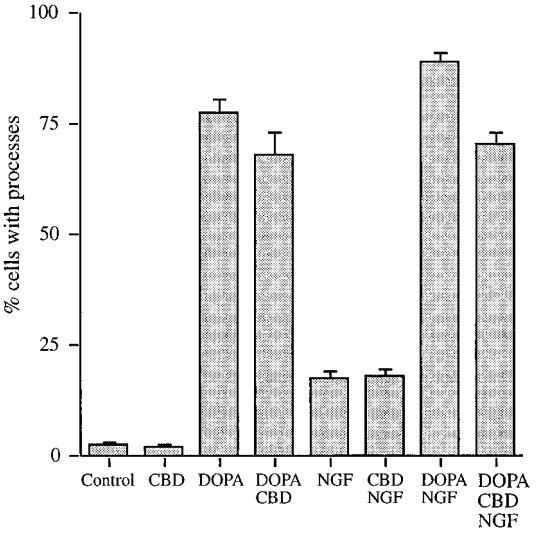

Fig. 6. Effects of L-DOPA and carbidopa on neurite induction. The percentage of cells that exhibited neurites after exposure to L-DOPA, carbidopa (*CBD*), and NGF in combination as in Fig. 5 are reported as mean ± standard error ($n = 10$ cultures). Carbidopa did not block process outgrowth evoked by either L-DOPA or NGF ($p < 0.001$, ANOVA); carbidopa and control groups were not different.

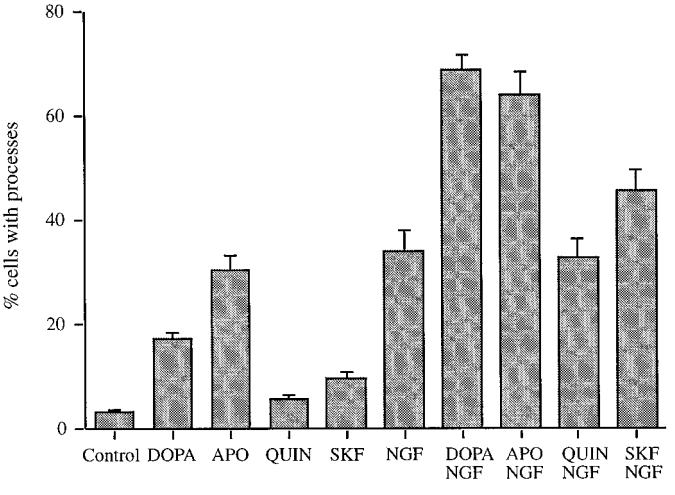

Fig. 7. Effect of DA receptor agonists on process outgrowth. The percentage of cells that exhibited neurites after exposure to 50 μM L-DOPA, 10 μM apomorphine, 80 μM quinpirole, 100 μM SKF38393-A, or 50 ng/ml NGF. Data are reported as mean ± standard error ($n = 6$ cultures). Of the DA receptor agonists examined, only apomorphine induced large-scale process outgrowth comparable with L-DOPA ($p < 0.001$ versus both control and NGF-only treatments, ANOVA followed by *post hoc* test), although there is a small but significant effect with SKF38393-A ($p < 0.01$ versus both control and NGF-only treatments).

populations, the nonparametric Komolgorov-Smirnov statistic (Van der Kloot, 1991) was used (GB-Stat; Dynamic Microsystems, Silver Spring, MD).

Results

Induction of neurites by L-DOPA. To test whether L-DOPA alone might induce neurite outgrowth of undifferentiated (i.e., NGF-naive) PC12 cells, we exposed the cultures to 12–200 μM L-DOPA alone for 24–48 hr. In all cases, untreated cultures contained ≤ 1% of cells with neurites. L-DOPA elevated the percentage of cells that exhibited neuritic processes at 50, 100, and 200 μM levels, although the affect was attenuated at the highest concentration (Fig. 1, *leftmost bars*). These neurites were relatively delicate in appearance and generally retracted about 3 days after the initial L-DOPA exposure, even in trials where L-DOPA levels were maintained. Between different sets of cultures, the percentage of cells that expressed temporary neurite induction varied from 20–75%. Therefore, all experiments reported used sister cultures derived from the same parent culture, which, as indicated in Fig. 1, showed low variance.

L-DOPA (24 hr) in combination with NGF synergistically increased the number of cells that exhibited processes compared with NGF treatment alone (Fig. 1, *rightmost bars*) as well as the length and arborization (see below). In contrast to treatments with L-DOPA alone that produced temporary neurite outgrowth, there was little variance between groups of sister cultures.

Because L-DOPA has been reported to produce apoptosis in PC12 cells (Walkinshaw and Waters, 1995), we examined the occurrence of apoptosis in undifferentiated PC12 cultures at 2–3 days after plating using the nuclear stain Hoechst 33342 (Fig. 2, *inset*). We found that 50 μM L-DOPA (48 hr) did not increase apoptotic profiles, whereas 100 μM L-DOPA increased apoptosis by nearly 30-fold (Fig. 2, *leftmost columns*). NGF (50 ng/ml) effectively blunted L-DOPA-induced apoptosis (Fig. 2, *rightmost columns*). In agreement with a previous study (Basma *et al.*, 1995), L-DOPA induced an overall dose-dependent decrease in cell number (Fig. 3, *leftmost columns*). This effect was also effectively blunted by NGF (Fig. 3, *rightmost columns*). Note that the total number of cells in groups treated with NGF was lower, because this factor differentiates the cells and halts mitosis.

L-DOPA-mediated cell death could be caused by elevated intracellular oxidative stress. To examine this, we adapted methods used previously with cultured midbrain dopamine neurons to detect intracellular oxyradicals caused by methamphetamine exposure (Cubells *et al.*, 1994). This approach uses DCF, a membrane-permeant fluorogenic compound that is trapped in living cells after de-esterification; in the presence of peroxides or hydroxy radicals, this compound is converted to a fluorescent derivative. DCF has recently been used to examine oxidative stress in PC12 cells using flow cytometry (Aoshima *et al.*, 1997). If oxyradicals are induced within neurons by L-DOPA, we would expect to see an increase in fluorescent cell bodies. Indeed, we found that a low percentage of the cells were labeled by DCF, and that the number of labeled cells increased 12-fold after high levels of L-DOPA (Table 1). The label generally appeared to have a granular distribution (not shown), similar to the pattern of label in cell bodies of midbrain dopamine neurons after methamphetamine treatment (Cubells *et al.*, 1994). Although NGF alone did not reduce the number of DCF-positive cells not exposed to L-DOPA, NGF inhibited intracellular oxidative stress observed after L-DOPA administration (Table 1). Because of striking effects on cell number, apoptosis, and intracellular oxidative stress observed with 200 μM L-DOPA, we chose to use 50 μM L-DOPA for our experimental protocols.

Long term L-DOPA exposure potentiates quantal DA release from neurites. To test whether L-DOPA induced functional presynaptic changes, we adapted recently developed techniques to measure quantal release of catecholamines directly from PC12 neurite varicosities (Zerby and Ewing, 1996). Preliminary observations indicated that quantal release from the NGF-naive processes was extremely rare (three events observed from one site; Davila V, unpublished observations), probably related to the destability and gradual loss of processes that occurred unless NGF was present.

After coexposure to NGF alone or NGF and L-DOPA, the electrodes were placed over process varicosities (Fig. 4, *inset*); quantal events were not observed along the neurites between the varicosities. Depolarization with high K^+ promoted quantal release from the varicosities (Fig. 4, A and B). Compared with cells exposed to NGF alone, cells exposed to L-DOPA (50 μM for 48 hr at 2 days after plating) and constant NGF released quanta composed of twice the number of molecules, even > 1 week after exogenous L-DOPA was washed out. The cumulative distribution of the cubed root of the quantal sizes, which is normally distributed (Finnegan *et al.*, 1996; Pothos *et al.*, 1996), demonstrates that the entire population of quanta was shifted to larger values (Fig. 4C). The mean interspike interval was ~2-fold shorter in duration in cells exposed to both L-DOPA and NGF (Table 2; Fig. 4D). Therefore, the frequency of release as well as quantal size

TABLE 2

Amperometric spike characteristics

Analysis of quantal release from neurite varicosities. Exposure of PC12 cells treated with NGF alone to 80 mM K^+ evoked 214 release events from nine varicosities of a total of 16 recorded (see Fig. 3, *inset*), whereas stimulation of cells treated with NGF and 50 μM L-DOPA evoked 439 release events from eight varicosities of a total of 15 recorded. Data in the table are expressed as mean per cell ± standard error. Analysis of the entire population of events, rather than cell means, showed similar trends (quantal size and interspike intervals of the population are shown in Fig. 4).

	No. of events	Interspike interval	SD/Mean	No. of molecules	i_{max}	Width	$t_{1/2}$
					pA	*msec*	
NGF (n = 9)	23 ± 12	571.1 ± 94.1	1.70 ± 0.15	111,000 ± 27,000	7.1 ± 1.7	13.9 ± 1.6	3.5 ± 0.4
NGF & L-DOPA (n = 8)	55 ± 12*	269.9 ± 68.4*	1.39 ± 0.10	226,000 ± 55,000*	9.8 ± 1.5	22.5 ± 7.6	4.2 ± 0.5

* $p < 0.01$ different from control group by Kolmogorov-Smirnov statistics.
i_{max}, maximum peak amplitude.
$t_{1/2}$, the width at half height.
SD Mean, standard deviation of the interspike interval divided by the mean interspike interval.

was increased by a single 48 hr exposure to L-DOPA. As the value of the standard deviation of the interspike interval divided by the mean interval was unchanged, L-DOPA did not elicit bursting release; rather, it reduced interspike intervals to produce a greater number of release events per stimulation. Together, the data indicate that L-DOPA potentiated release from NGF-treated varicosities by increasing quantal size, the number of quantal events (i.e., quantal content), and the release frequency.

DA is not required for neurite induction. The finding that acidic fibroblast growth factor and catecholamines synergistically up-regulate tyrosine hydroxylase activity in DA neurons in culture (Stull and Iacovitti, 1996) suggests that an analogous action by NGF and DA may occur after upregulation of DA levels because of exposure to L-DOPA. To test whether DA synthesis was required for L-DOPA-induced neurite outgrowth, we examined the effects of carbidopa (25 μM), which blocks DA synthesis at this exposure by inhibiting aromatic acid decarboxylase (Basma *et al.*, 1995; Mena *et al.*, 1997b).

Carbidopa itself did not induce neurite outgrowth (compare Fig. 5, A and C; Fig. 6) and did not block neurite outgrowth induced by L-DOPA (compare Fig. 5, B and D; Fig. 6). Carbidopa slightly (20%) inhibited neurite outgrowth induced by the combination of L-DOPA and NGF ($p < 0.05$; compare Fig. 5, F and H; Fig. 6). Therefore, synthesis of DA from L-DOPA was not required to induce neurite outgrowth, although there may be a small DA-mediated potentiation of combined NGF/L-DOPA induction.

It has been noted that the tyrosine kinase inhibitor genistein blocks NGF-mediated attenuation of peroxide accumulation and excitotoxicity in hippocampal cultures (Mattson *et al.*, 1995). As G protein-linked receptors, DA receptors might be expected to either stimulate tyrosine kinase or potentiate the tyrosine kinase activity induced by neurotrophin binding. However, genistein (10 μM for 24 hr) had no effect on process outgrowth elicited by either NGF alone (50 ng/ml) or L-DOPA/NGF (12–200 μM; data not shown).

To further exclude the possibility that the synergistic responses to L-DOPA/NGF were mediated by DA, we examined the effects of DA receptor ligands on NGF-induced neurite outgrowth. Consistent with the interpretation that DA itself is not required for L-DOPA induction of process outgrowth, we observed that 24 hr exposure of PC12 cultures to the D_2 agonist quinpirole did not induce neurites, whereas the DA agonist SKF 38393-A produced a small but significant potentiation of outgrowth (Fig. 7); similar results were observed at 48 hr exposures (not shown). Moreover, the D_2 antagonist sulpiride (20–200 μM) did not block L-DOPA effects (data not shown), confirming that activation of at least one of these receptors was not required.

Surprisingly, only the D_1/D_2 agonist apomorphine (10 μM) had a potent synergistic effect on neurite outgrowth similar to that of L-DOPA (Fig. 7). However, there was no synergistic effect on NGF-elicited outgrowth using a combination of quinpirole and SKF 38393-A (data not shown), which indicates that the effect of apomorphine was not caused by binding to both classes of DA receptors. We noted that of the DA receptor ligands tested, apomorphine was the only one to induce a markedly visible extracellular pigment, in this case a greenish pigment reminiscent of the brownish pigment of L-DOPA quinone oxidation products. To examine this product, we measured relative extracellular pigments levels using spectrophotometric readings of the medium at 490 nm (a blue wavelength), which has been used previously to measure the brown pigment (Mena *et al.*, 1992; Mena *et al.*, 1993) and was seen here to clearly measure the greenish pigment (Table 3). Although indirect, the correlation of quinone formation and potentiation of NGF-induced outgrowth suggests that the synergistic effect could be attributable to an oxyradical-mediated mechanism. Because apomorphine and other compounds that act as mild pro-oxidants are known to stimulate GSH synthesis in several cell types (Han *et al.*, 1996), we examined the effect of apomorphine on GSH levels (Table 4). We found that apomorphine, as well as NGF, indeed elevated the presence of GSH in the cultures.

Effects of elevated GSH synthesis and synthesis inhibition. From the above experiments, it seemed reasonable that an alternate pathway that could underlie neurite initiation by L-DOPA might occur via stimulation of reactive oxygen species derived from L-DOPA metabolism. To examine if antioxidants might promote neurite outgrowth, we

TABLE 3

Induction of extracellular quinones

Five days after plating, cultures were exposed to 50 μM L-DOPA, 500 μM LNAC, 50 ng/ml NGF, or 10 μM apomorphine as indicated for 24 hr. The extracellular media were then assayed for quinone levels. Data are mean ± standard error ($n = 6$). These measurements were run with two batches of sister cultures; in each case, $n = 6$ cultures per condition.

	Quinone (absorbance $\times 10^3$)	Quinone (absorbance $\times 10^3$)
Controls	116 ± 5 (100%)	118 ± 14 (100%)
L-DOPA	238 ± 8[a] (205%)	ND
NGF	115 ± 7 (99%)	99 ± 6 (84%)
L-DOPA & NGF	216 ± 8[a] (186%)	ND
LNAC	107 ± 5 (92%)	ND
L-DOPA & LNAC	123 ± 6[d] (106%)	ND
Apomorphine	ND	176 ± 3[a] (149%)
Apomorphine & NGF	ND	149 ± 3[b,c] (126%)

[a] $p < 0.001$ versus controls.
[b] $p < 0.01$ versus controls.
[c] $p < 0.001$ for cultures treated with apomorphine/NGF versus apomorphine.
[d] $p < 0.001$ for cultures treated with L-DOPA/LNAC versus L-DOPA.

TABLE 4

Effects of apomorphine and NGF on GSH synthesis

Five days after plating, cultures were exposed to 50 μM L-DOPA, 10 μM apomorphine, or 50 ng/ml NGF for 24 hr. The cultures were then assayed for total GSH and protein levels. Data are mean ± standard error ($n = 6$).

	GSH	Protein	GSH
	μg/well		*μg/mg protein*
Controls	1.3 ± 0.04 (100%)	615 ± 4.4 (100%)	2.11 ± 0.02 (100%)
Apomorphine	16.41 ± 0.8* (1262%)	523 ± 2.0 (92%)	31.37 ± 0.9* (1487%)
NGF	2.19 ± 0.2* (169%)	701 ± 7.0** (114%)	3.12 ± 0.1* (148%)
Apomorphine & NGF	18.1 ± 0.5 (1391%)	547 ± 2.0 (89%)	33.10 ± 1.20 (1569%)

* $p < 0.001$ versus controls.
** $p < 0.01$ versus controls.

used LNAC, a hydrophilic antioxidant (Yan *et al.*, 1995) that also elevates intracellular GSH in PC12 cells (Ferrari *et al.*, 1995; Yan *et al.*, 1995; Kranich *et al.*, 1996). We found that LNAC and L-DOPA elevated GSH levels in a manner similar to that of NGF and apomorphine (Table 5). Consistent with a general neurotrophic effect by antioxidant actions, LNAC also potentiated L-DOPA-induced temporary neurite formation at levels identical to those of NGF (Fig. 8).

To test whether GSH synthesis *per se* was required by L-DOPA/NGF synergistic effects, we used BSO, which inhibits the GSH synthetic enzyme gamma-glutamylcysteine synthase. We found that 10 μM BSO treatment had no effect on total protein levels, which indicates that this level of exposure was relatively nontoxic but decreased GSH to 45% of control values (Table 6). At 24 hr, BSO had no effect on the temporary induction of neurites by L-DOPA, NGF, or combined NGF/L-DOPA (Fig. 9A). (In some cases, we noted that BSO elicited short, temporary neurites and we noted a small ($< 10\%$) and temporary potentiation of the effects on NGF and NGF/DOPA at 48 hr; data not shown). However, at 5 days, a time point when NGF alone has nearly caught up with the synergistic effects of NGF/L-DOPA, BSO decreased neurite outgrowth elicited by both NGF and NGF/L-DOPA (Fig. 9B).

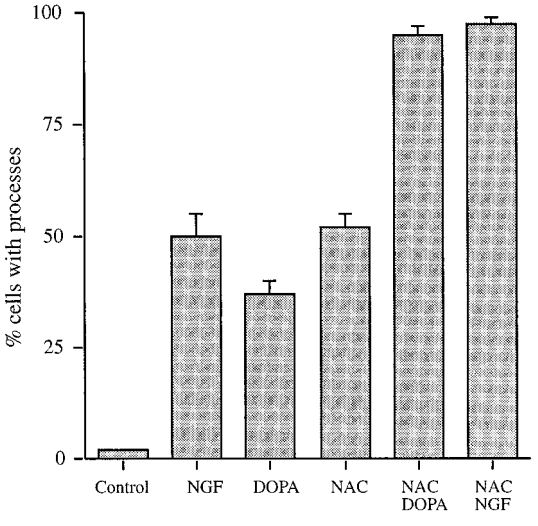

Fig. 8. Effect of LNAC on process outgrowth. The percentage of cells that exhibited neurites following exposure to LNAC, L-DOPA, and NGF in combination. Data are reported as mean ± standard error ($n = 10$ cultures). PC12 cultures at 2 days after plating were treated for 3 days with vehicle, 50 μM L-DOPA, or 500 μM LNAC with or without NGF (50 ng/ml). LNAC induced process outgrowth similarly to L-DOPA (all categories $p < 0.001$ versus control) and potentiated the effects of NGF and LNAC ($p < 0.001$, ANOVA followed by *post hoc* test).

Discussion

L-DOPA, a widely prescribed intervention used to treat Parkinson's disease and related disorders, has both trophic and toxic effects. Recently, factors that are produced by glia have been implicated in the compound's neurotrophic effects (Mena *et al.*, 1997b), which suggests that release of an L-DOPA- or L-DOPA metabolite-elicited neurotrophic factor may be involved. To examine that possibility, we have used the PC12 cell line, which expresses neurites on exposure to NGF. Strikingly, L-DOPA provided a synergistic effect on the process outgrowth induced by this neurotrophic factor. A physiologically relevant result of this neurotrophic effect was that L-DOPA enhanced the effect of NGF on quantal neurotransmitter release from the neurite varicosities. The findings suggest that long-lasting effects of L-DOPA may play an important role in the induction of neurite outgrowth and enhanced presynaptic function because of effects on neurotrophic factor-mediated pathways.

Oxidative metabolism and L-DOPA-induced neurite outgrowth. L-DOPA alone induced unstable neurites in PC12 cells that usually retracted within 3 days unless NGF was present. Interestingly, we observed similar small, temporary processes after exposure to LNAC, BSO, and apomorphine, all compounds that can promote relatively mild oxidant effects. This suggests that a molecular pathway initiated by mild pro-oxidant treatment can induce a form of early process outgrowth, but that subsequent steps, likely triggered by tyrosine receptor kinase receptor binding, are required for neurite maintenance. Mild pro-oxidant treatments have been suggested to protect a range of cells from death by up-regulating GSH (Han *et al.*, 1996). The effect we have observed on neurite outgrowth may stem from the action of L-DOPA or an L-DOPA metabolite directly on NGF signaling, analogous to the observation that H_2O_2 generation is required for signal transduction by platelet-derived growth factor (Sundaresan *et al.*, 1995). Alternatively, antioxidant mechanisms that result from NGF binding [elevation of catalase expression, for instance (Sampath *et al.*, 1994)], may be potentiated by L-DOPA. Indeed, our data indicate that GSH was up-regulated not only by NGF but also by all of the treatments that potentiated neurite outgrowth, including L-DOPA, LNAC, and apomorphine, as well these compounds in combination. However, the full elucidation of the biochemical pathways and roles played by oxyradical pathways in the

TABLE 5

Effects of L-DOPA, LNAC, and NGF on GSH synthesis

Five days after plating, cultures were exposed to 50 μM L-DOPA, 500 μM LNAC, or 50 ng/ml NGF for 24 hr. The cultures were then assayed for total GSH and protein levels. Data are mean ± standard error ($n = 6$).

	GSH	Protein	GSH
	μg/well		*μg/mg protein*
Controls	1.68 ± 0.2 (100%)	993 ± 13 (100%)	1.69 ± 0.08 (100%)
L-DOPA	3.17 ± 0.1* (190%)	737 ± 18* (74%)	3.02 ± 0.06* (179%)
NGF	2.81 ± 0.2* (169%)	1270 ± 12* (128%)	2.21 ± 0.08* (131%)
L-DOPA & NGF	5.63 ± 0.01*† (339%)	1460 ± 70*† (147%)	3.86 ± 0.01*† (228%)
LNAC	4.17 ± 0.2* (249%)	1290 ± 80* (130%)	3.23 ± 0.1* (191%)
L-DOPA & LNAC	5.03 ± 0.1*† (300%)	1183 ± 60** (119%)	4.22 ± 0.1*† (250%)

* $p < 0.001$ versus controls.
** $p < 0.01$ versus controls.
† $p < 0.001$ for cultures treated with L-DOPA & NGF versus L-DOPA, or treated with L-DOPA & LNAC versus L-DOPA.

synergistic response between L-DOPA and NGF will require further study.

As in midbrain neurons (Mena *et al.*, 1997a) L-DOPA-induced outgrowth in PC12 cells did not depend on conversion to DA, because the effects were independent of blockade of DA synthesis by carbidopa, were not blocked by the D2-like antagonist sulpiride, and similar responses were not observed after exposure to DA receptor agonists, with the exception of apomorphine. However, apomorphine is itself a pro-oxidant, as seen by its metabolism to a quinone. We suggest that apomorphine may act in a manner similar to L-DOPA, as a mild pro-oxidant that stimulates a cascade of downstream effects that may be synergistic with neurotrophin activation. However, although GSH seemed to be required for maintenance of L-DOPA-induced neurite outgrowth in both neurons and PC12 cells, this effect was delayed in PC12 cells. Another difference between findings for these cell types is that midbrain DA neurons required unidentified glial factors for the L-DOPA-induced neurotrophic action, as indicated by the requirement for glial-conditioned medium, while L-DOPA-induced PC12 neurite outgrowth was strongly potentiated by NGF alone. The differences may be consistent with the different biological milieus of the cell types. In the ventral midbrain, DA neurons are surrounded by glia (Damier *et al.*, 1993) that provide a range of compounds and actions that mediate oxyradical pathways, including catalase activity, GSH, monoamine oxidase B activity, and other mechanisms to sequester and degrade toxic compounds (Tsacopoulos and Magistretti, 1996), including control of potentially neurotoxic ambient glutamate levels via glial conversion to glutamine (Rosenberg and Aizenman, 1989). However, immortalized cell lines such as the adrenal chromaffin tumor PC12 cell line have acquired characteristics that allow them to thrive independently of the factors contributed by other cell types.

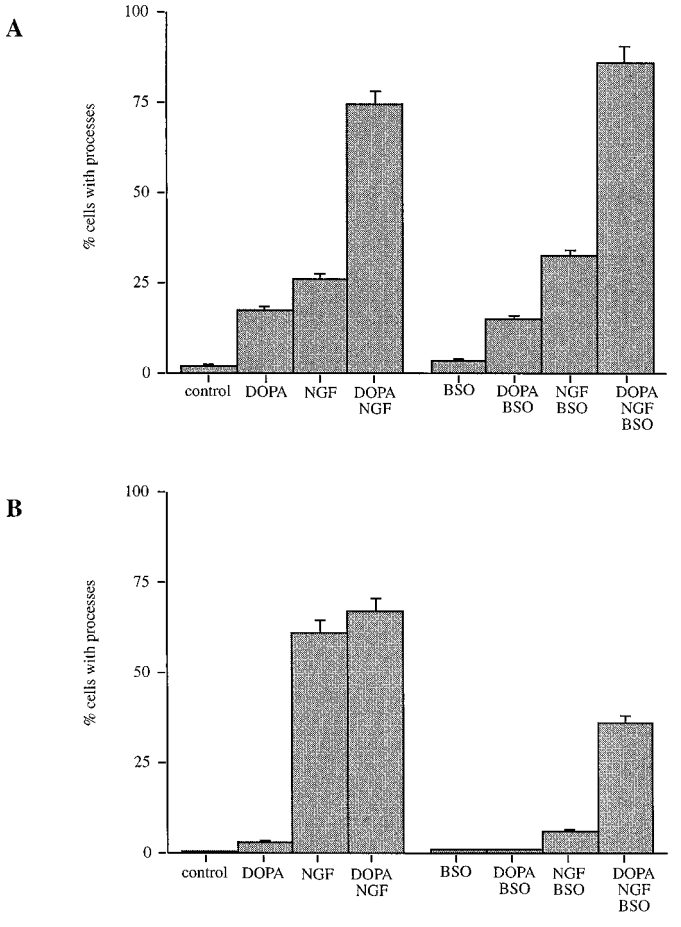

Fig. 9. Effect of BSO on L-DOPA and NGF-dependent process outgrowth. PC12 cultures at 2 days after plating were treated for 24 hr or 5 days with vehicle, 10 μM BSO, or 50 μM L-DOPA, with or without NGF (50 ng/ml). The percentage of cells that exhibited neurites are reported as mean ± standard error (A, $n = 4$ cultures; B, $n = 6$ cultures). A, At 24 hr, each intervention presented increased neurites over control ($p < 0.01$, ANOVA followed by *post hoc* test). BSO slightly potentiated the effects of NGF ($p < 0.05$) and NGF with DOPA ($P < 0.05$). B, At 5 days, L-DOPA, NGF, and L-DOPA with NGF each presented increased neurites over control ($p < 0.01$, ANOVA followed by *post hoc* test). BSO alone was not different from controls but reduced the presence of neurites when included with L-DOPA ($p < 0.001$), NGF ($p < 0.001$), and L-DOPA with NGF ($p = 0.026$).

L-DOPA promotes the quantal release of DA from neurites. To examine neurotransmitter release from neurite varicosities, as opposed to total release from the culture, we used new electrochemical approaches that measure quantal catecholamine secretion in real time. We found that in PC12 cells, long term L-DOPA increased the quantal release rate, the number of quanta per stimulation, and the quantal size of neurotransmitter release. This contrasts with short term (30 min) L-DOPA exposure, where quantal size is potentiated but the frequency of release is unchanged (Pothos *et al.*, 1996). The elevated quantal size is presumably caused by augmented vesicular DA levels that persist even after more than 1 week of L-DOPA withdrawal. The elevated release rate could be caused by a range of mechanisms, including the presence of more vesicles in the varicosities, a larger percentage of vesicles docked to the plasma membrane, or effects on calcium channels or other proteins involved in exocytosis. Indeed, an alteration in ion channel expression may be consistent with the finding that long term L-DOPA administration *in vivo* increases the firing rate of midbrain DA neuron action potentials (Harden and Grace, 1995). It is possible that a trophic mechanism similar to that which promotes

TABLE 6

Effects of L-DOPA and BSO on GSH synthesis

Five days after plating, cultures were exposed to 50 μM L-DOPA or 10 μM BSO for 24 hr. The cultures were then assayed for total GSH and protein levels. Data are mean ± standard error ($n = 6$).

	GSH	Protein	GSH
	μg/well		*μg/mg protein*
Controls	1.3 ± 0.04 (100%)	615 ± 4.4 (100%)	2.11 ± 0.02 (100%)
L-DOPA	3.07 ± 0.2* (236%)	609 ± 18 (99%)	5.04 ± 0.1* (239%)
BSO	0.58 ± 0.01* (45%)	640 ± 8 (104%)	0.91 ± 0.01* (43%)
L-DOPA & BSO	0.78 ± 0.01** (60%)	590 ± 17 (96%)	1.32 ± 0.01** (62%)

* $p < 0.001$ versus controls.
** $p < 0.001$ for cultures treated with L-DOPA versus L-DOPA & BSO.

neurite outgrowth may also be trophic for the presynaptic sites or synthesis of secretory granules and associated proteins. However, the salient point is that the elevated neurite outgrowth after the synergistic response to L-DOPA and NGF was physiologically significant, providing an impressive elevation of transmitter release at individual varicosities.

The long term effects of L-DOPA explored here may be relevant for treatment of Parkinson's disease because of the important issue of whether L-DOPA improves prognosis. L-DOPA has not been observed to promote toxicity using *in vivo* models, and normal primates treated with high doses of L-DOPA for 3 months exhibit an intact nigrostriatal tract with no evidence of oxidative damage to lipids, proteins, or DNA (Jenner, 1996). Nevertheless, L-DOPA may be toxic for diseased neurons already prone to degeneration. If L-DOPA normally contributes to DA release in the striatum in part by promoting neurite outgrowth and potentiating presynaptic mechanisms of neurotransmitter release, it may be worth considering additional therapy with LNAC, which is effective *in vivo* (Zimmerman *et al.*, 1989) and has been used safely in humans for more than 30 years (Aruoma *et al.*, 1989).

In summary, long term exposure to L-DOPA seems to increase release of DA by at least two mechanisms: potentiating the process outgrowth induced by trophic factors and increasing quantal release from neurite varicosities. The ability of other compounds that elicit subneurotoxic oxidative stress to provide a similar trophic effect indicates that oxidative metabolism may play an important role in the potentiation of response to neurotrophic factors. A consequence of this may include promoting mechanisms that protect sites that contain monoaminergic synaptic vesicles from local oxidative stress.

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