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# Methamphetamine Neurotoxicity Involves Vacuolation of Endocytic Organelles and Dopamine-Dependent Intracellular Oxidative Stress

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**Methamphetamine (MA) produces selective degeneration of dopamine (DA) neuron terminals without cell body loss. While excitatory amino acids (EAAs) contribute to MA toxicity, terminal loss is not characteristic of excitotoxic lesions nor is excitotoxicity selective for DA fibers; rather, EAAs may modulate MA-induced DA turnover, suggesting that DA-dependent events play a key role in MA neurotoxicity. To examine this possibility, we used postnatal ventral midbrain DA neuron cultures maintained under continuous EAA blockade. As *in vivo*, MA caused neurite degeneration but minimal cell death. We found that MA is a vacuologenic weak base that induces swelling of endocytic compartments; MA also induces blebbing of the plasma membrane. However, these morphological changes occurred in MA-treated cultures lacking DA neurons. Therefore, while collapse of endosomal and lysosomal pH gradients and vacuolation may contribute to MA neurotoxicity, this does not explain selective DA terminal degeneration. Alternatively, MA could exert its neurotoxic effects by collapsing synaptic vesicle proton gradients and redistributing DA from synaptic vesicles to the cytoplasm. This could cause the formation of DA-derived free radicals and reactive metabolites. To test whether MA induces oxidative stress within living DA neurons, we used 2,7-dichlorofluorescin diacetate (DCF), an indicator of intracellular hydroperoxide production. MA dramatically increased the number of DCF-labeled cells in ventral midbrain cultures, which contain about 30% DA neurons, but not in nucleus accumbens cultures, which do not contain DA neurons. In the DA neuron cultures, intracellular DDF labeling was localized to axonal varicosities, blebs, and endocytic organelles. These results suggest that MA redistributes DA from the reducing environment within synaptic vesicles to extravesicular oxidizing environments, thus generating oxygen radicals and reactive metabolites within DA neurons that may trigger selective DA terminal loss.**

***[Key words: ammonium chloride, amphetamine, cell culture, 2,7-dichlorofluorescin diacetate, dopamine, hydrogen peroxide, nucleus accumbens, oxygen radicals, oxidative stress, superoxide dismutase, ventral tegmental area]***

The psychostimulant methamphetamine (MA) is commonly abused for its rewarding effects, which are thought to result from dopamine (DA) release in the nucleus accumbens (nAcc; Cho, 1990; Wise and Hoffman, 1992). MA administration also induces a selective degeneration of striatal DA terminals (Seiden and Ricaurte, 1987). This effect appears to be DA dependent since it is attenuated by inhibition of DA synthesis (Gibb and Kogan, 1979; Fuller and Hemrick-Luecke, 1982; Schmidt et al., 1985). However, NMDA receptor antagonists also reduce MA neurotoxicity (Sonsalla et al., 1989, 1991). Recent studies employing *in vivo* microdialysis have linked the protective effect of MK-801 to the ability of this drug to diminish MA-induced striatal DA overflow (Marshall et al., 1993). Similarly, decreases in striatal DA overflow appear to underlie the protective effect of $D_1$ and $D_2$ antagonists (O'Dell et al., 1993). Thus, several strategies for reducing MA neurotoxicity appear to share as a common mechanism the attenuation of MA-induced DA release, arguing for the importance of DA itself as a key mediator of MA neurotoxicity.

DA readily oxidizes at physiological pH to form potentially toxic metabolites including hydroxyl radical, superoxide radical, hydrogen peroxide, and quinones (Graham et al., 1978; Cohen, 1984; Graham, 1984; Slivka and Cohen, 1985). Seiden and Vosmer (1984) originally suggested a direct role for DA-dependent oxidation in MA neurotoxicity by demonstrating the presence of a compound chromatographically indistinguishable from 6-hydroxydopamine (6-OHDA) in the brain after MA administration. Although this finding remains controversial (Rollema et al., 1986; Fuller, 1991), the observation that antioxidants such as ascorbate and vitamin E partially antagonize MA neurotoxicity (De Vito and Wagner, 1989) provides further evidence that MA neurotoxicity could involve oxidative stress.

Amphetamines are weak bases that collapse intracellular pH gradients in acidic organelles that include lysosomes, endosomes, and synaptic vesicles (Sulzer and Rayport, 1990). Acidotropic uptake of MA could cause osmotic swelling (Sulzer and Holtzman, 1989) and explain the vacuole formation seen in cultured neurons exposed to MA (Cubells et al., 1991; Bennett et al., 1993). Furthermore, since amphetamines displace DA from synaptic vesicles to the cytoplasm (Sulzer and Rayport, 1990; Sulzer et al., 1992a,b) MA might promote intraneuronal DA-dependent oxidative stress. Normally, synaptic vesicles

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maintain catecholamines in a reduced state due to their acidic pH and probable high levels of ascorbate (Beers et al., 1986; Njus et al., 1986; Johnson, 1988). If displaced to the cytoplasm, DA would likely undergo both auto-oxidation and enzymatic oxidative metabolism by monoamine oxidase (MAO), leading to the formation of hydrogen peroxide, free radicals, and quinones (Graham et al., 1978; Slivka and Cohen, 1985). In this article, we show that MA exerts both of these potentially neurotoxic effects: (1) a swelling of endocytic organelles that occurs in all neurons as well as glia, and (2) a redistribution of DA from reducing to oxidizing environments that leads to DA-dependent oxidative stress within neurons. We thus propose that DA-dependent oxidative stress may be the initial event in MA neurotoxicity.

A preliminary presentation of this work has been made in abstract form (Cubells et al., 1991).

## Materials and Methods

*Cell culture.* Culture techniques followed those previously described (Rayport et al., 1992) with the addition of glutamate antagonists. Briefly, postnatal day 1–3 rat pups were anesthetized with 5 mg of ketamine intraperitoneally followed by hypothermia. A ventral midbrain segment including both the ventral tegmental area (VTA) and substantia nigra (SN) was dissected from a 2-mm-thick coronal slice at the level of the midbrain flexure; the nAcc was dissected from a horizontal slice at the level of the anterior commissure. The regions of interest were cut into 1 $mm^3$ cubes and enzymatically dissociated with papain (Worthington; 10 U/ml) in an oxygenated bicarbonate-buffered saline (cf. Baughman et al., 1991) for 2 hr at 32°C. The tissue cubes were triturated and the resulting cell suspension plated onto monolayers of cortical astrocytes. Individual cultures contained about 20,000 neurons and ventral midbrain cultures contained about 5 pmol of DA (Sulzer et al., 1993). Cultures were maintained at 35°C in 5% $CO_2$ in serum-free media supplemented with 1% calf serum (Hyclone), containing 0.5 mM kynurenate (a level that antagonizes both NMDA and AMPA receptors; Watkins and Collingridge, 1989), 0.25% albumin, 0.5 mM glutamine, 100 μg/ml transferrin, 15 μM putrescine, 30 nM $Na_2SeO_3$, 30 nM $T_3$, 25 μg/ml insulin, 200 nM progesterone, 125 nM cortisol, 5 μg/ml superoxide dismutase, and 10 μg/ml catalase (all from Sigma). Proliferation of non-neuronal cells was suppressed by addition of 25 μM 5-fluorodeoxyuridine with 70 μM uridine (Sigma) to the medium 1 d after plating. MA was introduced directly into the kynurenate-containing culture medium by dilution of a 1000× stock for 10 and 100 μM MA exposures or of a 100× stock for 500 and 1000 μM MA exposures. Radioimmunoassay (High Specificity Methamphetamine Abuscreen system, Roche Diagnostics) showed that MA concentrations in the medium were constant over 6 d (the maximum exposure duration).

*Viability assays.* Calcein-acetoxymethyl ester (calcein AM; 3.00 μM) and ethidium homodimer-1 (3.75 μM) were used to identify living and dead cells, respectively (Live/Dead Assay, Molecular Probes). Calcein AM is membrane-permeable dye that is cleaved by intracellular esterases to produce an impermeant green-wavelength fluorophore in living cells, while ethidium homodimer is a red-wavelength fluorophore that selectively permeates the broken membranes of dying cells and stains their nuclei. To assess neuronal viability, cultures were stained for 75 min at 32°C with the two dyes, rinsed with phosphate-buffered saline, and examined under fluorescein epifluorescence. This assay clearly distinguished living from dead neurons as all neurons were labeled and none were double labeled. The frequency of dead neurons in each treatment was compared to that expected on the basis of the control using $\chi^2$ tests. There were no differences between treatments in total numbers of neurons per field (control dishes had 32.9 neurons per 25× field, while dishes exposed to the maximum 1000 μM dose of MA for 6 d had 32.5 neurons per 25× field; $t = 0.07$, df = 25, $p > 0.9$), showing that the viability assays were not skewed by disintegration of dead cells over the course of the experiment.

*Quantitative analysis of MA-induced neurite loss.* DA neurons and their processes were visualized by tyrosine hydroxylase (TH) immunocytochemistry using standard methods (Rayport et al., 1992). The first 10 $TH^+$ neurons encountered in a random scan of each dish were evaluated for neurite arborization by an observer blind to the treatment condition. Processes of $TH^+$ neurons were quantified by placing each cell body in the center of a 40× scope field (radius of 250 μm) and counting the number of primary neurites that reached, or whose branches reached, the perimeter of the field. Experiments were performed in matched sets of sister cultures and were repeated in five to seven culture dishes per treatment group. Data reported were from two groups of cultures prepared on different days. Frequency distributions for each treatment group were generated by assigning each neuron to a bin corresponding to 0, 1, 2, 3, or ≥4 neurites extending to the field perimeter (fewer than 3% of neurons had >4 neurites). These values are discrete nominals and were analyzed by nonparametric statistics. Contingency table analysis of the pooled data (Dowdy and Wearden, 1983) rejected the null hypothesis that the arborization index distributions were homogeneous across treatments ($\chi^2 = 51.68$, df = 16; $p < 0.001$). Distributions were then compared between individual treatments using $\chi^2$ goodness-of-fit tests (Zar, 1984).

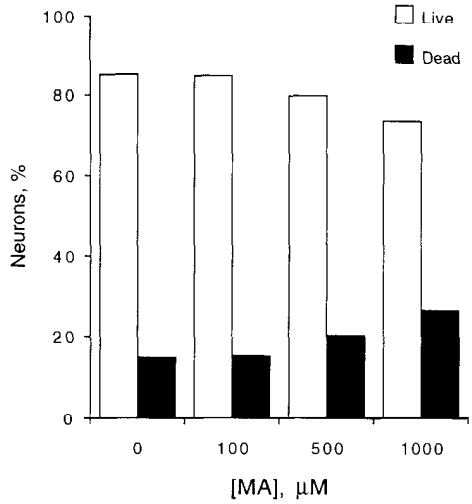


*Figure 1.* MA produces limited cell death in ventral midbrain culture. Cultures were exposed to MA from day 10 through day 15 *in vitro*; MA levels were shown to be stable by radioimmunoassay (data not shown). Live and dead cells were counted after labeling with calcein AM and ethidium homodimer. The highest exposure to MA (1 mM) increased the percentage of dead neurons from 15% to 26% ($\chi^2 = 46.9$, df = 1, $p < 0.001$, compared to that expected on the basis of the control). Exposure to 100 μM or 500 μM MA did not produce significant increases in frequencies of dead neurons ($\chi^2 = 0.062$ and 0.355, df = 1, respectively; $n = 426$–$487$ neurons per condition).

*Endocytic labeling.* Cultures were exposed to green fluorescent latex microspheres (1:1000; Luma-Fluor) for 18–24 hr. Cultures were then rinsed with physiological saline and control images of several fields recorded. MA or other weak bases were added for 1–3 hr and images of the same fields rerecorded.

*DCF spectrofluorometry.* Aliquots of 1 mM 2,7-dichlorofluorescin diacetate (DCF; Molecular Probes) were prepared in ethanol and stored at −85°C. To determine if DA auto-oxidation increased DCF fluorescence, DCF was first deesterified by incubating 0.5 ml of 1 mM DCF in 2 ml of 0.01 *N* NaOH at 20°C for 30 min, after which the pH was neutralized with 10 ml of 50 mM Tris, pH 7.2 (Cathcart et al., 1983). DA at a final concentration of 0, 1, or 10 mM was added to the deesterified DCF, 150 μM final concentration, in 1.5 ml of 50 mM Tris, pH 7.2. The increase in fluorescence over time was measured at room temperature with a spectrofluorometer (Varian SF-330) set at an excitation wavelength of 505 nm and an emission wavelength of 525 nm.

*DCF in neuronal culture.* Aliquots of 10 mM DCF were prepared in dimethyl sulfoxide and stored at −85°C. Immediately before use, an aliquot was diluted to 1 μM in 32°C minimal essential medium. Cultures were incubated for 15 min and then rinsed twice with oxygenated physiological saline (135 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.3) at 32°C. To avoid photodynamic damage, the excitation beam was attenuated with a 10% neutral density filter and low-light images recorded using a chilled CCD camera (Photometrics); digital images were stored to disk and processed

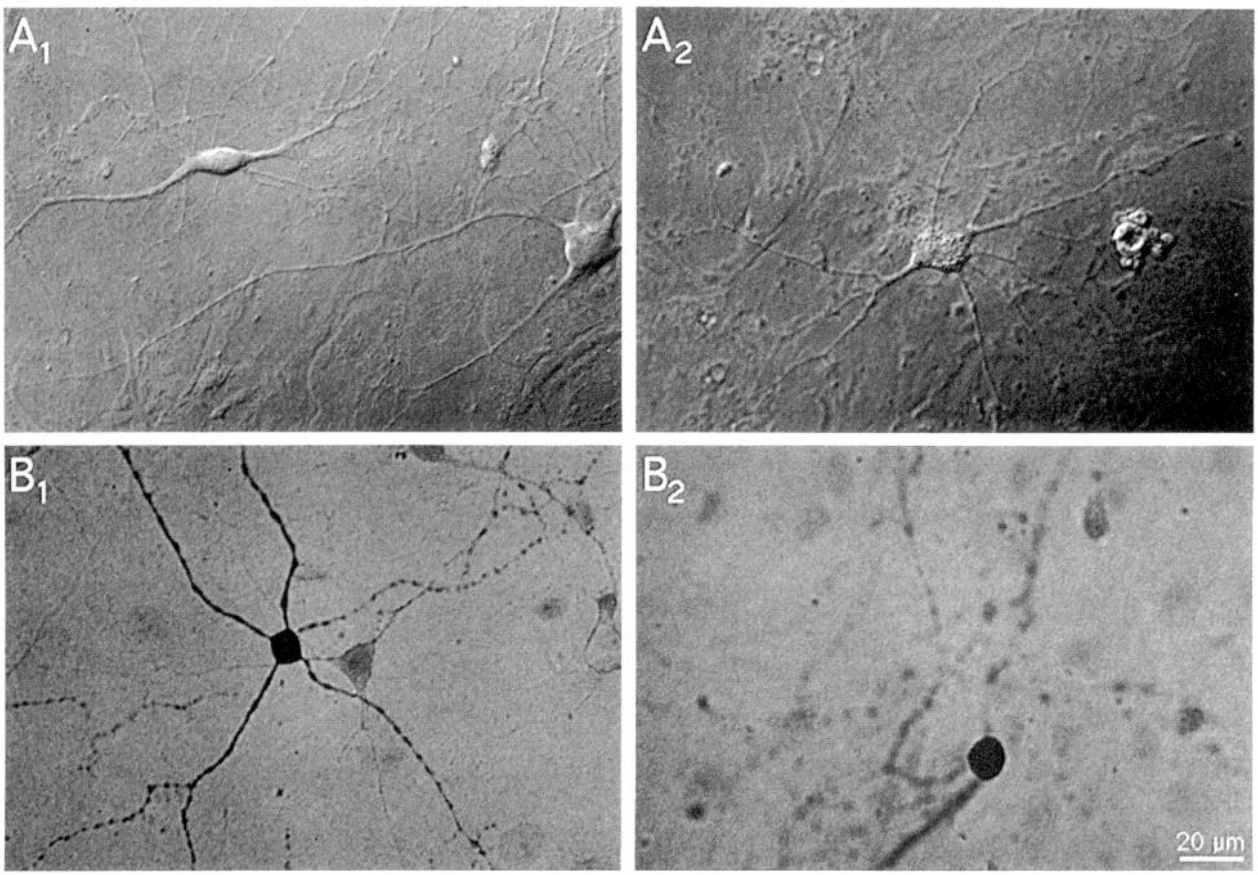


*Figure 2.* MA produces neurite degeneration, vacuolation, and blebs. $A_1$, Nomarski image of representative neurons from control ventral midbrain cultures. Note the relatively smooth appearance of the cell body and long, thin processes. $A_2$, Nomarski image of representative neurons from ventral midbrain cultures treated with 500 µM MA for 1 d. Note the vacuolated appearance of the cell body and the swollen processes of the neuron on the left. The neuron on the *right* has apparently lost its processes and displays several blebs; such cells were still alive by fluorescence viability assay. Such cells are identified as neurons because neurites can be observed at intermediate stages of degeneration (compare Fig. 8) and because oligodendrocytes are both rare and identifiable by morphological criteria. $B_1$, A representative DA neuron from a control culture stained for TH. Note the long processes including a putative axon studded with varicosities. Such varicosities contain DA synaptic vesicles and mitochondria (Sulzer and Rayport, 1990; Rayport et al., 1992). $B_2$, DA neuron from a culture stained for TH after exposure to 100 µM MA for 6 d. Note the remnants of processes, which were not visible under Nomarski optics prior to TH staining, suggestive of cytoskeletal collapse.

using NIH IMAGE (Wayne Rasband; available on Internet via anonymous FTP from zippy.nih.nimh.gov in the directory /pub/nih-image). Labeled and unlabeled neurons were counted by a second observer blind to both the treatment and the cellular origin of each culture. A minimum of 300 neurons per condition were counted. To assess whether imaged cells were dopaminergic, the fields examined were circled with a diamond objective marker, cultures were processed for TH immunocytochemistry, and the images were correlated.

## Results

### *Morphological effects of MA exposure*

*In vivo*, MA causes degeneration of DA processes but not DA cell bodies (Ricaurte et al., 1982, 1984). Similarly, in postnatal ventral midbrain cultures maintained in 0.5 mM kynurenate, MA caused no reduction in numbers of viable neurons except at suprapharmacologic concentrations above 500 µM (Fig. 1); however, profound morphological changes were evident both in neurons and glia. Under Nomarski optics, neurons in control cultures appeared relatively smooth (Fig. 2*A*), whereas numerous spherical cytoplasmic structures imparted a rough, grainy appearance to MA-treated neurons (Fig. 2*B*). These *vacuoles* were visible in all MA-treated neurons and glia, even after exposures of cultures to as little as 10 µM MA for 1 d or to 100 µM MA for 2 hr. Under phase-contrast optics, the MA-induced vacuoles were identical in appearance to those observed by Bennett et al. (1993); they also resembled weak base-induced structures in retinal neurons (Sulzer and Holtzman, 1989). Larger ovoid structures, often protruding above the surfaces of cell bodies and processes, frequently appeared in neurons (but not in glia) after MA exposure (Fig. 2*B*) and are referred to as *blebs*. Although neuronal blebs were common in MA-treated cultures, they were also apparent in neurons in unhealthy control dishes (not shown). Thus, unlike vacuoles, they were not specifically associated with MA exposure. As shown below, vacuoles and blebs are distinct structures, rather than the same structures at different stages of evolution, as we initially proposed (Cubells et al., 1991).

MA produced swelling and degeneration of DA neuronal processes (Fig. 2; see also Fig. 8). In culture, DA neurons characteristically elaborate processes extending considerable distances, with axon-like neurites often extending over a millimeter (Rayport et al., 1992). After exposure to MA for 6 d, there was a marked loss of DA neuron processes as visualized by TH immunocytochemistry. Counts of the number of neurites extending more than 250 µM from their parent cell bodies confirmed

quantitatively that MA produced widespread process degeneration in a concentration-dependent manner. Incubation in either 100 or 500 μM MA for 6 d produced a dose-dependent increase in the relative frequencies of neurons exhibiting only 1 or 0 neurites reaching the 250 μM perimeter (Fig. 3; $p < 0.01$ either for MA concentration compared to control, or for 100 μM MA compared to 500 μM MA).

*Acidotropic uptake of MA*

In the course of the viability assays, we observed that both vacuoles and blebs characteristically excluded the cytosolic vital stain calcein AM, suggesting that these structures were membrane delimited. Since amphiphilic weak bases such as ammonia (added as $NH_4Cl$) produce osmotic swelling after accumulating in acidic organelles, particularly endosomes and lysosomes (Okhuma and Poole, 1981; Sulzer and Holtzman, 1989), we hypothesized that vacuoles and blebs might arise from these organelles due to acidotropic uptake of MA.

To test this, we labeled endocytic organelles with fluorescent latex microspheres. The microspheres, which are taken up endocytically into lysosomes (Egensperger and Hollånder, 1988), produced a punctate pattern of fluorescence in both neurons and glia in control cultures (Figs. 4, 5), similar to that seen with the weak base vital dye acridine orange (Sulzer and Rayport, 1990; Sulzer et al., 1993). In MA-treated cultures, vacuoles were microsphere labeled (Fig. 4) while blebs were never labeled.

Since vacuolation also occurred in astrocytes, we took advantage of their two-dimensional morphology and large endocytic compartments to compare the effects of MA and other weak bases (Okhuma and Poole, 1981; Cain and Murphy, 1986; Sulzer and Holtzman, 1989). Individual astrocytes were identified after uptake of fluorescent latex microspheres, imaged, exposed to the test compounds, and reimaged. MA produced increases in the diameter of endocytic organelles similar to the prototypic vacuologenic weak base ammonia (added as ammonium chloride), whereas the nonvacuologenic weak base tributylamine did not (Fig. 5), demonstrating that MA is a vacuologenic weak base.

*MA-induced oxygen radical formation*

To examine whether MA promotes oxidative stress in cultured neurons, we used DCF, a membrane-permeant fluorogenic compound that is trapped in living cells following deesterification by cytoplasmic enzymes. In the presence of hydroperoxides or hydroxyl radicals, 2,7-dichlorofluorescin (deesterified DCF) is converted to the fluorescent compound 2,7,-dichlorofluorescein (Cathcart et al., 1983). If cytoplasmic DA levels increase, we would expect to see increased production of hydroperoxides or free radicals following MA administration.

To determine if DCF responds to DA auto-oxidation, we measured the fluorescence of deesterified DCF in the presence of 0, 1, and 10 mM DA. Fluorescence increased in a time- and concentration-dependent manner (Fig. 6), demonstrating that DCF detects DA auto-oxidation products. Since hydrogen peroxide increases DCF fluorescence within seconds (Cathcart et al., 1983), the slow buildup of fluorescence likely reflects DA breakdown.

In control VTA cultures incubated with DCF, fewer than 1% of the neurons were brightly labeled, whereas labeled neurons were common after MA. Figure 7 shows Nomarski and fluorescent images of a group of ventral midbrain neurons incubated for 12 hr in 100 μM MA and then with DCF. The lower-most

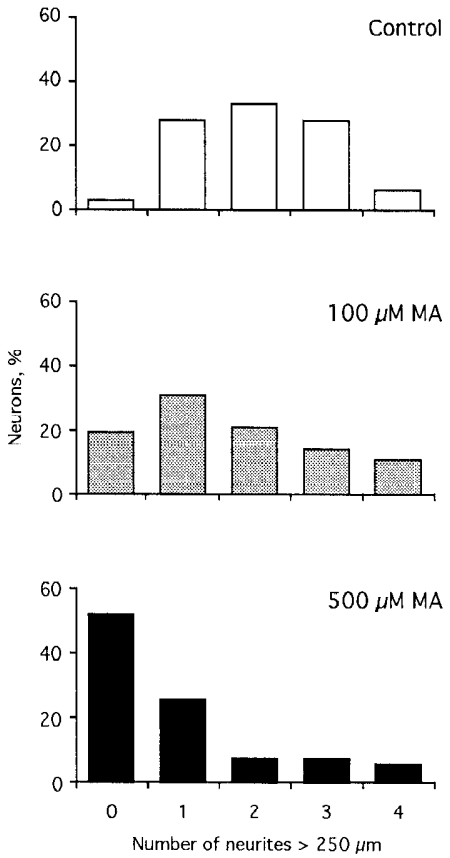


*Figure 3.* Quantification of process loss from DA neurons following MA exposure. Cultures were exposed to 100 or 500 μM MA for 6 d and immunostained for TH. DA neurons were then classified by the number of neurites that extended beyond a 250 μm radius. Relative frequencies of MA-treated DA neurons classified in this way differed significantly from those expected on the basis of the control sample (100 μM: $\chi^2 = 13.89$, df = 5, $p < 0.01$; 500 μM: $\chi^2 = 102.5$, df = 5, $p < 0.001$). In addition, the 500 μM group exhibited a different frequency distribution than did the 100 μM group ($\chi^2 = 33.4$, df = 5, $p < 0.001$). As shown in Figure 2, those differences reflected MA-induced reductions in both the number and extent of $TH^+$ neurites.

neuron shows diffuse cytoplasmic labeling with brighter spots of fluorescence localized in an endocytic pattern. The heterogeneity of DCF fluorescence apparent in this group of neurons was typical of that observed in MA-treated ventral midbrain cultures. Astrocytes showed minimal staining. Discrete intracellular DCF labeling was also apparent in neurites. In a representative DCF-labeled VTA neuron after 48 hr in 10 μM MA, several blebs were visible along the main neurite (presumably a swollen axon since it extended over 500 μm) as well as vacuoles within the cell body (Fig. 8). All of the blebs, as well as many of the vacuoles, were prominently DCF labeled, demonstrating the presence of high levels of hydroperoxides or oxygen radicals within these presumably MA-induced structures. This pattern of intense punctate DCF labeling was never observed in control cultures.

*MA induces oxidative stress selectively in DA neuron cultures*

If the MA-induced increase in oxidative stress is DA mediated, MA should promote hydroperoxide formation only in cultures of DA-containing neurons. To examine this, we conducted preliminary experiments using the TH inhibitor α-methyl-*p*-tyrosine; however, we found that it also alkalinized intracellular

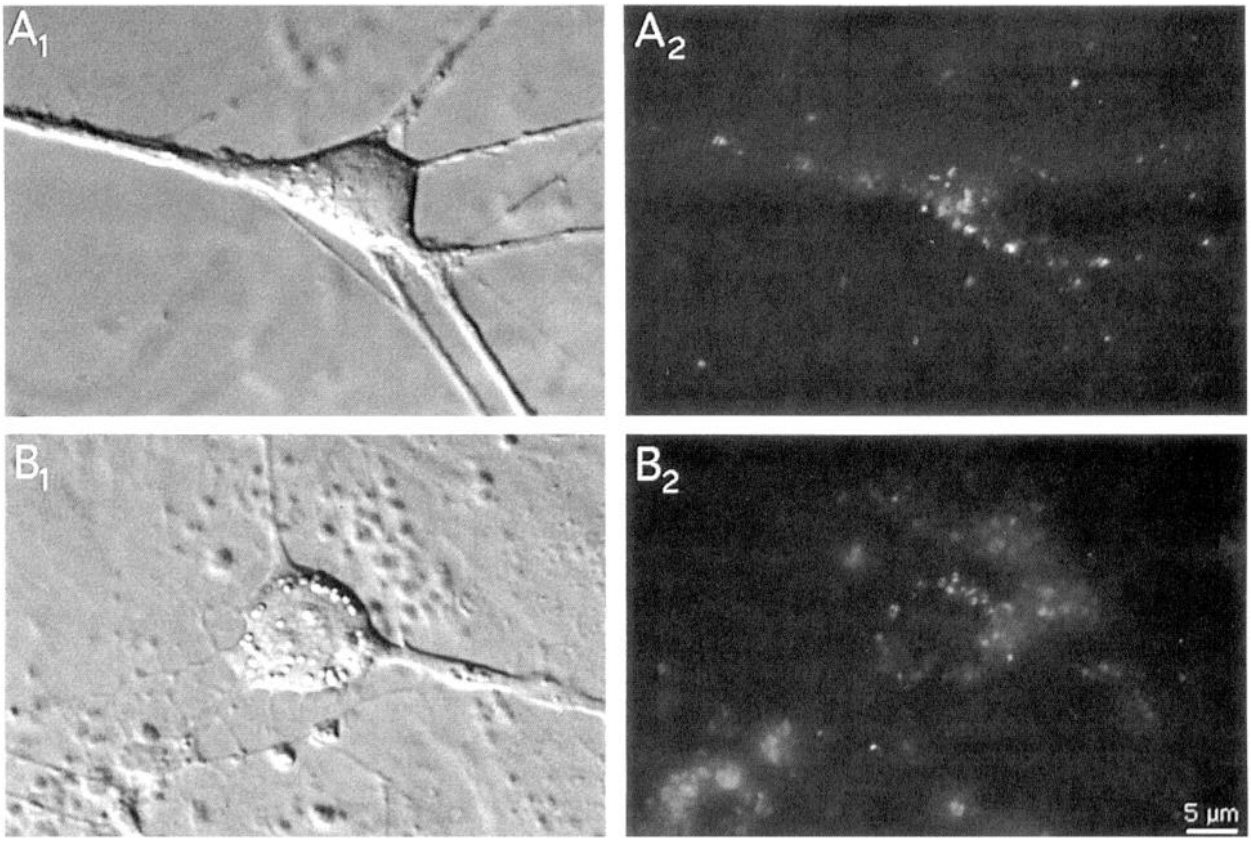


*Figure 4.* MA causes vacuolation of endocytic compartments. $A_1$, A representative neuron from a control ventral midbrain culture appears relatively smooth under Nomarski optics. $A_2$, Under fluorescein optics, endocytic compartments are identified in the same control cell using fluorescent latex microspheres as an endocytic label. $B_1$, A representative neuron from another ventral midbrain culture exposed to 500 μM MA for 24 hr. Prominent vacuoles are seen in both the neuron and in the glial monolayer. $B_2$, Under fluorescein optics, many of the vacuoles in B1 are labeled by microspheres, thus demonstrating that they arise from endocytic organelles. In contrast, blebs were never microsphere labeled (not shown), suggesting they do not arise from endocytic structures.

acidic compartments. We therefore compared VTA cultures (approximately 30% DA neurons) to control cultures from their principal target area, the nAcc (0% DA neurons). The cultures contained similar densities of neurons, were derived from the same animals, and were treated in parallel. Neurons in VTA or nAcc cultures showed low rates of neuronal DCF labeling in the absence of MA (Fig. 9). After 48 hr incubation in 10 μM MA, VTA cultures exhibited dramatically higher proportions of fluorescent neurons, whereas no increase occurred in nAcc cultures (Fig. 9; $p < 0.001$; see caption for statistical details).

To determine if non-DA neurons in VTA cultures also undergo increased oxidative stress, VTA cultures were treated with MA and subsequently processed to identify DA neurons by TH antigenicity. After 10 μM MA for 2 d, a significantly greater proportion of DA neurons (40%, 14 of 35) were DCF labeled than of neurons overall (25%, 25 of 101; $\chi^2 = 5.87$, df = 1, $p < 0.025$; Fig. 10).

## Discussion

To focus on the role of DA in MA neurotoxicity, we have examined the effects of MA on postnatal mesolimbic DA neuron cultures maintained under continuous excitatory amino acid (EAA) receptor blockade. We found that MA is a vacuologenic weak base, accounting for its propensity to induce vacuolation of endocytic organelles. In addition, alkalization of acidic intracellular gradients may have a range of deleterious effects on cellular metabolism. However, neither of these effects are DA dependent. In contrast, MA induces a DA-dependent increase in intracellular oxidative stress at discrete intracellular sites, particularly in neuronal processes, which may be the initial step in MA neurotoxicity.

### *Validity of model system*

The neurotoxic effects of MA in EAA-blocked postnatal mesolimbic cultures resemble those seen in more intact preparations. Several studies have examined the morphological consequences of MA administration *in vivo*. Ricaurte et al. (1982, 1984) showed that MA induces degeneration of ascending DA axons in the nigrostriatal and mesoaccumbens projections but no reduction in the number of neuronal cell bodies in the SN or VTA. Selective MA-induced process degeneration has been confirmed in a range of studies (Ellison et al., 1978; Lorez, 1981; Hess et al., 1990; cf. Dawirs et al., 1991). The selectivity of MA neurotoxicity for neuronal processes is consistent with the observations of Kontur et al. (1987, 1991), who found MA-induced depletion of DA levels in embryonic midbrain reaggregate cultures after 100 μM for 7 d, but no decrement in the number of DA cell bodies. As seen *in vivo*, we found that DA neurons exposed to MA for 6 d in culture exhibited a dose-dependent loss of neurites but a low incidence of cell death. This obser-

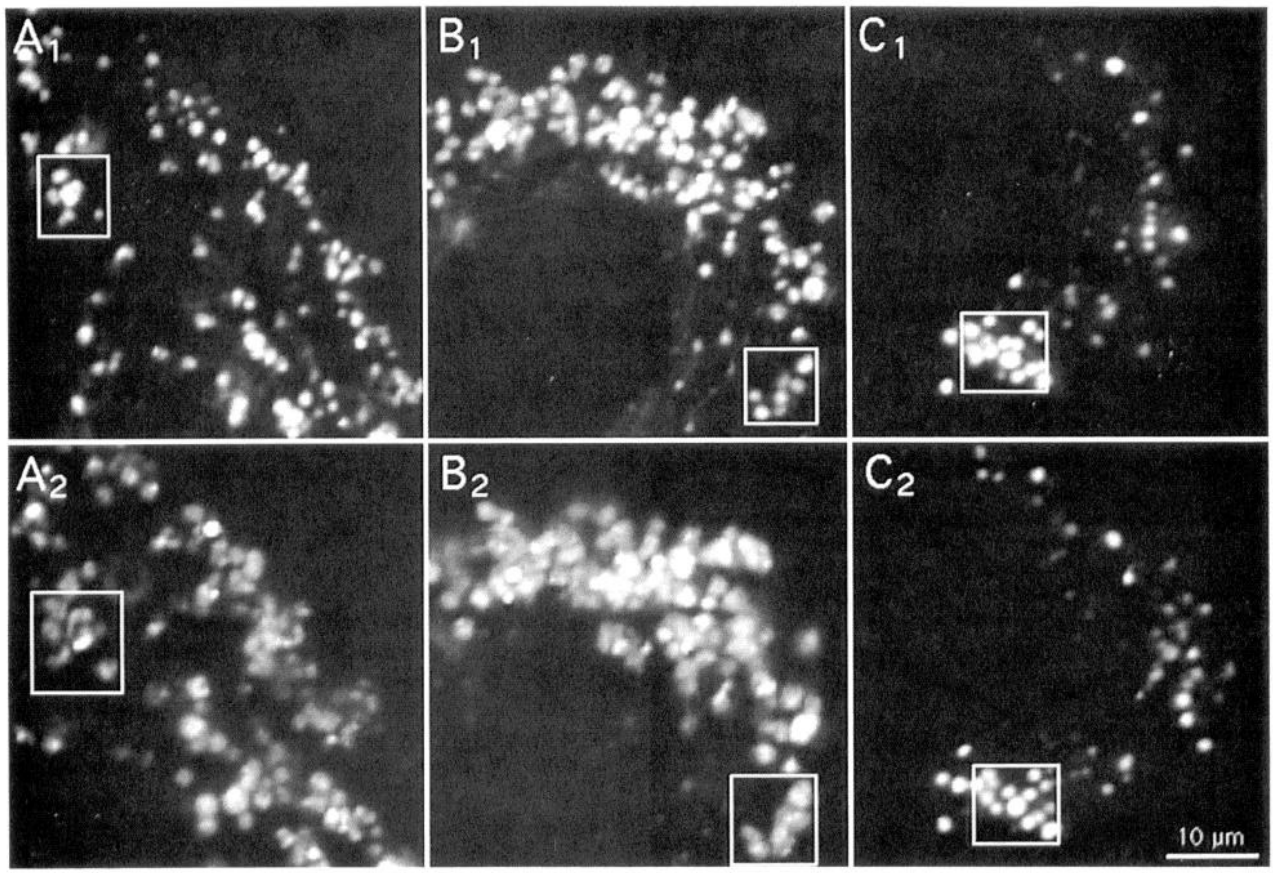


*Figure 5.* Amphiphilic weak bases cause vacuolation of endocytic organelles. Astrocytes, like neurons, show vacuolation with weak base exposure; because of their flat morphology, changes in the size of endocytic compartments can be followed easily. Each pair of micrographs shows an area of the same cortical astrocyte before and after exposure to weak bases. For comparison, *rectangles* delineate clusters of the same organelles. $A_1$, $B_1$, and $C_1$, Astrocytes from sister cultures labeled by uptake of fluorescent microspheres, prior to the addition of the weak bases. $A_2$, MA (500 μM for 1.5 hr) induces swelling of labeled endocytic compartments. $B_2$, The classic vacuologenic weak base ammonia (added as 10 mM $NH_4Cl$ for 2.5 hr) also causes marked swelling. $C_2$, In contrast, the lipophilic weak base tributylamine (10 mM for 2.5 hr) does not cause swelling, although at this concentration it abolishes intracellular pH gradients (Sulzer et al., 1993).

vation provides a useful criterion for *in vitro* neurotoxicity induced by neuropharmacologically relevant levels of MA.

In our experiments, significant neuronal mortality did not occur except in the presence of concentrations of MA higher than those that arise in the brain (≥500 μM) with standard pharmacologic doses (10–100 μM after dosages of 1–10 mg/kg in the rat; Melega et al., 1992), although possibly in the range of concentrations reached in the brains of tolerant abusers during binges (Baselt, 1982). The kynurenate in the culture medium may have exerted a neuroprotective effect; whole-cell, patch-clamp observations in midbrain cultures (D. Sulzer and S. Rayport, unpublished observations) confirm that this concentration of kynurenate blocks both NMDA and AMPA-type glutamate receptors. In addition, extracellular superoxide dismutase or catalase in our culture medium may have imparted an increased tolerance to MA exposure.

### *Neurotoxicity associated with acidotropic MA uptake*

Acidotropic uptake of MA would be expected to increase the osmotic gradient in acidic organelles causing an influx of water, and producing vacuolation identical to that seen with the classic weak base ammonia (Okhuma and Poole, 1981; Sulzer and Holtzman, 1989). We found that both MA- and ammonium-induced swelling occurred specifically in endocytic organelles.

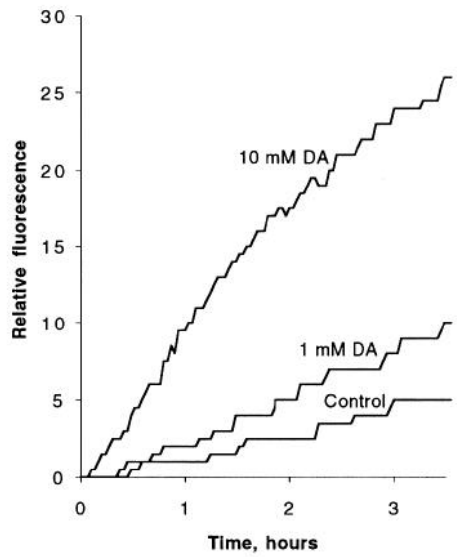


*Figure 6.* Fluorescent detection of DA auto-oxidation products *in vitro*. DCF was deesterified and its fluorescence followed in a spectrophotometer for 3 hr (excitation λ = 505 nm; emission λ = 525 nm). The slow increase in DCF fluorescence was accelerated by DA in a concentration-dependent manner, probably reflecting the rate of DA auto-oxidation.

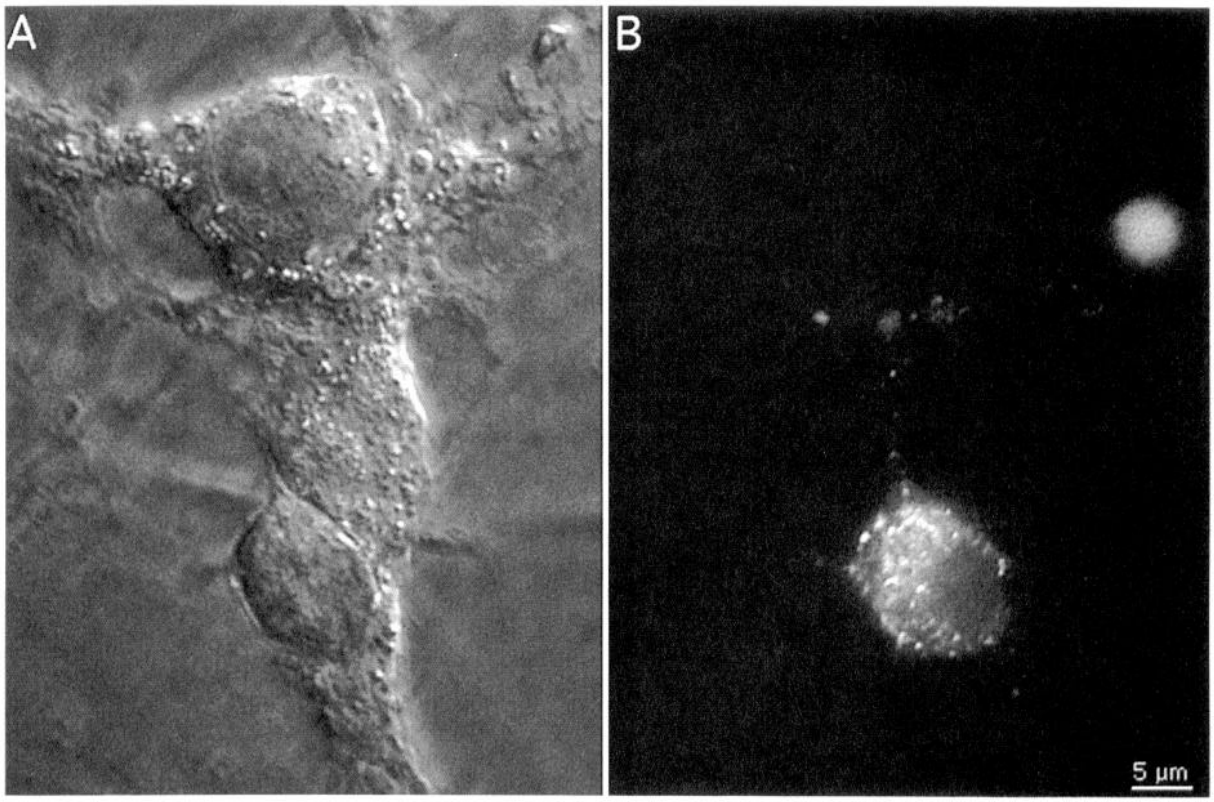


*Figure 7.* Intracellular oxidative stress due to MA. *A*, A cluster of living ventral midbrain neurons were exposed to 100 μM MA for 12 hr. Vacuolation is evident under Nomarski optics. *B*, The same field under fluorescein optics shows that only one neuron is labeled by DCF. The distribution is strikingly similar to that of endocytic organelles (compare Fig. 4). Some structures within the glial monolayer are lightly labeled. In control cultures, fewer than 1% of neurons were brightly labeled; about 6% neurons showed light labeling (compare Fig. 9).

In contrast, tributylamine, a nonvacuologenic weak base that is not trapped because it remains lipophilic in its protonated form, did not produce vacuolation. If lysosomal swelling resulted in leakage of degradative enzymes into the cytoplasm, as has been reported in gout and silicosis (Kane et al., 1980; cf. De Duve et al., 1974), vacuolation could play a direct role in MA toxicity. However, we never observed release of the endocytic tracer to the cytosol, arguing against large-scale lysosomal leakage. Other effects following alkalization of intracellular acidic compartments could disrupt pH-dependent metabolic functions vital to cellular function including protein glycosylation, membrane transport, and uptake of growth factors. Similar conditions have been suggested to occur in cystic fibrosis, perhaps due to the impaired acidification in endocytic organelles and Golgi (Barasch et al., 1991). However, these effects are not specific to DA neurons and are probably too slow to account for the relatively rapid toxicity seen with MA.

We suspect that vacuolation per se does not induce MA neurotoxicity since both vacuologenic weak bases such as chloroquine and nonvacuologenic weak bases such as tributylamine similarly inhibit fibroblast growth (Cain and Murphy, 1986). However, alkalization-induced lysosomal storage disorders (Kodavanti and Mehendale, 1990) could contribute nonspecifically to MA neurotoxicity. Lysosomal alkalization by psychostimulants might also account for lipofuscin-containing *spherical inclusions* seen after administration of methylenedioxymethamphetamine to primates (Ricaurte and McCann, 1992).

*MA neurotoxicity due to DA redistribution and oxidative stress*

DA has long been thought to play a role in MA neurotoxicity. Blocking DA synthesis protects against MA toxicity (Gibb and Kogan, 1979; Fuller and Hemrick-Luecke, 1982; Schmidt et al., 1985). DA uptake blockers antagonize MA toxicity (Marek et al., 1990; Fuller, 1991), possibly by reducing MA uptake (Zaczek et al., 1991) or by inhibiting MA-induced reverse transport of DA or DA metabolites (Sulzer et al., 1993). DA oxidation has been implicated in MA toxicity by experiments showing that *in vivo* administration of antioxidants such as ascorbate and vitamin E inhibit MA-induced reduction in brain levels of DA (De Vito and Wagner, 1989), and because catalase and superoxide dismutase, enzymes that metabolize hydrogen peroxide and superoxide, respectively, reduce toxicity due to elevated levels of extracellular catecholamines in cortical cultures (Rosenberg, 1988).

How MA interacts with intracellular DA pools directly bears on mechanisms of neurotoxicity. As opposed to the exchange diffusion model of amphetamine action (Fischer and Cho, 1979; Liang and Rutledge, 1982), where amphetamine would reduce cytoplasmic DA levels, in the weak base model amphetamines disrupt the electrochemical gradient that provides energy for monoamine accumulation in synaptic vesicles, leading to a redistribution of DA to the cytosol (Sulzer and Rayport, 1990; Sulzer et al., 1992b). Catecholamines more readily oxidize under cytoplasmic conditions (neutral pH, low ascorbate levels, and availability to MAO) than under intravesicular conditions (low

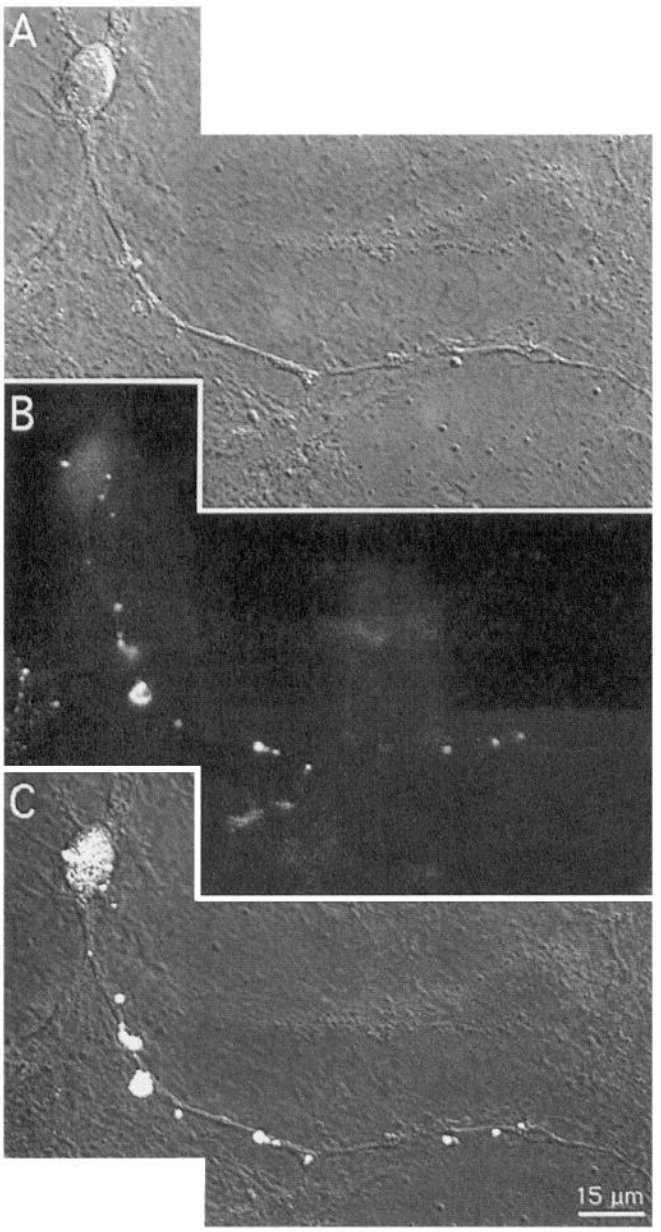


*Figure 8.* Discrete sites of MA-induced oxidative stress in neuronal processes. *A*, Under Nomarski optics, a neuronal process from a ventral midbrain culture exposed to 10 μM MA for 48 hr is swollen, particularly at varicosities, and has several blebs. *B*, Under fluorescein optics, DCF label is seen at discrete sites in the cell body and process. *C*, Superposition of *A* and *B* with digital enhancement of the DCF fluorescence shows the presence of label in most blebs and varicosities, demonstrating localized oxidative stress within the process. In the cell body, DCF labeling is present in a punctate distribution similar in pattern to the distribution of endocytic organelles as well as diffusely in the cytoplasm.

pH, high ascorbate levels, and no exposure to MAO). DA auto-oxidation leads to formation of reactive quinone derivatives, hydrogen peroxide, and free radicals (Graham et al., 1978; Slivka and Cohen, 1985). Therefore, after overwhelming cytoplasmic antioxidant systems, DA redistributed to the cytosol could increase oxidative stress.

We have shown that DA in solution undergoes auto-oxidation, resulting in the production of hydroperoxides or free radicals that oxidize the hydroperoxide-sensitive vital probe DCF

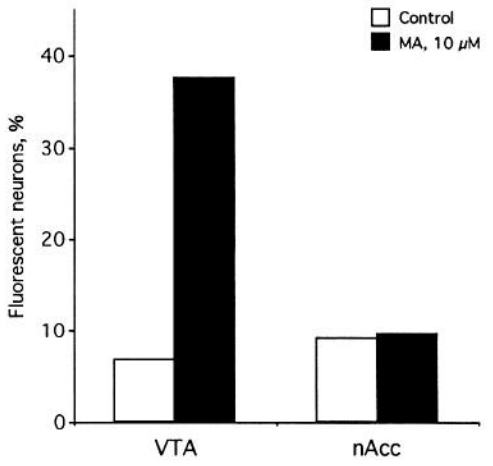


*Figure 9.* MA-induced oxidative stress is DA dependent. *A*, Sister VTA cultures (~30% DA neurons) and nAcc cultures (0% DA neurons) were treated with 10 μM MA for 48 hr. MA-treated VTA cultures showed a fivefold increase in the proportion of neurons labeled by DCF compared to control ($\chi^2 = 432.9$, df = 1, $p \ll 0.001$), whereas the nAcc cultures showed no MA-induced increase ($\chi^2 = 0.035$, df = 1, NS). An observer blind to treatment condition counted total and DCF-positive neurons; $n = 900$ neurons in three sister cultures per condition. The experiment was repeated three times with similar results.

to its fluorescent form. DCF has been used previously to detect intracellular production of hydroperoxides in non-neural cell types (Cathcart et al., 1983; Saito et al., 1992) and in a neuroblastoma-retinal hybrid cell line (Murphy et al., 1989). Applying DCF to neuronal culture, we demonstrate for the first time the presence of discrete intracellular sites of oxidative stress in neurons. These observations directly implicate intracellular compartments within aminergic neurons as important sites of MA-induced production of free radicals, in addition to the previously suggested extracellular sites (Seiden and Ricaurte, 1987), and bear out a prediction recently made by Fuller (1991) that MA may induce intracellular oxidative stress.

The varicosities in putative axonal processes are prominent *hot spots* of MA-induced hydroperoxides. This observation suggests a basis for the differential sensitivity of DA fibers to MA toxicity *in vivo*. Varicosities contain numerous mitochondria and are densely packed with synaptic vesicles that accumulate 5-hydroxydopamine (Arluison et al., 1978; Sulzer and Rayport, 1990; Rayport et al., 1992). Redistribution of DA from synaptic vesicles could overwhelm cytosolic antioxidant systems in axonal varicosities, leading to local DA auto-oxidation. MAO in the numerous mitochondria present in varicosities might contribute further to local oxidative stress by catalyzing the stoichiometric formation of hydrogen peroxide during the conversion of DA to DOPAC (Cooper et al., 1991). However, MAO inhibitors are not effective antagonists of MA neurotoxicity (Fuller, 1991), and MA itself inhibits MAO at high concentrations (Leitz and Stefano, 1971). Thus, intact MAO activity per se does not appear to be a prerequisite for MA neurotoxicity.

MA-induced DCF labeling in the cell body mostly appears in a distribution identical to that of endocytic organelles (i.e., endosomes and lysosomes). Lighter and more diffuse cytoplasmic labeling is also seen. The accumulation of label into endocytic organelles is probably due to lipophilic uptake since it appears to occur far too rapidly to reflect extensive endocytosis. Once inside lysosomes, deesterified DCF (dichlorofluorescin) might also selectively accumulate due to lysosomal esterases.

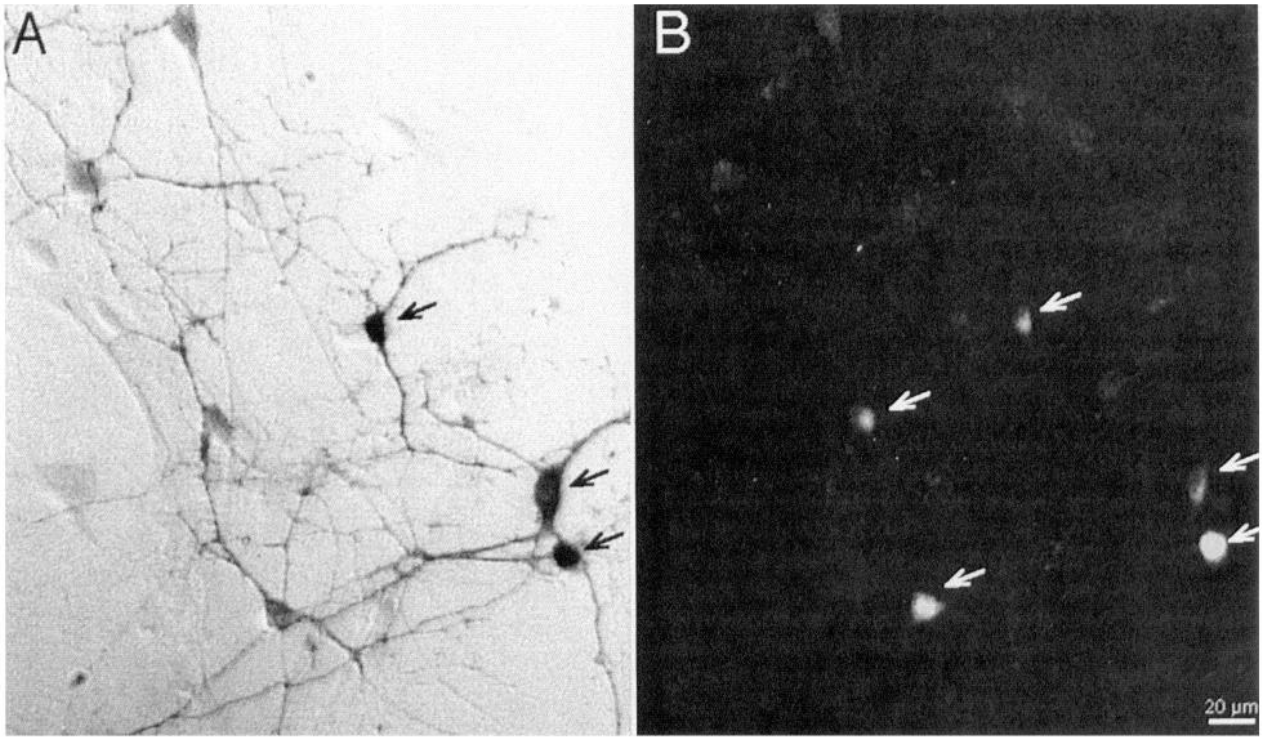


*Figure 10.* In ventral midbrain cultures, both DA and non-DA neurons undergo MA-induced oxidative stress. *A*, In a culture treated with 10 μM MA for 48 hr, TH immunocytochemistry shows that three cells in the field are DA neurons (*arrows*). Note the elaboration of varicosity-studded processes that appear to contact TH-negative neurons. *B*, When the same field, prior to fixation, is examined after DCF application, five neurons are labeled by DCF (*arrows*), two brightly so. The labeled neurons include all three DA neurons; the labeled non-DA neurons appear to be in contact with DA varicosities. Unlabeled neurons show low levels of fluorescence, which at higher magnification does not appear to be localized to intracellular structures.

Alternatively, MA-induced alkalization might promote formation of high levels of DA-derived free radicals and hydroperoxides within lysosomes and other acidic compartments, perhaps because the hydroxyl radical is a weak acid (pK = 4.7) most reactive in its protonated form (cf. Cohen, 1984).

DA appears to translocate into the cytosol and extracellular space within minutes after amphetamine administration (Sulzer et al. 1992a, 1993) while DCF oxidation is apparent within seconds in the presence of hydroperoxides (Cathcart et al., 1983). However, we noticed increases in DCF fluorescence within ventral midbrain cultures only after 12–24 hr of exposure to 10 μM MA (data not shown), with maximal increases in DCF fluorescence occurring 48 hr post-MA. One possible explanation for this delay is that intracellular antioxidants such as glutathione (Murphy et al., 1991) mitigate DA oxidation within aminergic neurons such that DA-derived reactive metabolites must accumulate with continued exposure to MA until they overwhelm the buffering capacity of intracellular antioxidant systems.

### *Cellular consequences of oxidative stress*

Our results demonstrate that pharmacologically relevant levels of MA increase local DA-dependent oxidative stress. Intracellular reactive metabolites are likely to degrade cellular components, including mitochondria (Wallace, 1992) and microtubules that are important for axonal cytoskeletal maintenance. Mechanisms explaining MA-induced damage to dendrites are less clear. We have not observed dendritic DA synaptic vesicles in culture (Sulzer and Rayport, 1990; Rayport et al., 1992) but dendrites may store DA in cisternal organelles (Cheramy et al., 1981). Degeneration of dendrites might depend on local DA stores, distribution of DA uptake sites, high surface area to volume ratio, or susceptibility of cytoskeletal elements to oxidative damage.

Based on the selective DCF labeling of DA neuron cultures, MA-induced formation of hydroperoxides or oxygen radicals appears to be DA dependent. However, non-DA neurons within the MA-treated DA cultures also exhibit DA-dependent increases in DCF labeling. Often DCF-labeled non-DA neurons receive afferents from DA neurons (see Fig. 10), suggesting that release of DA or DA metabolites could induce transynaptic oxidative stress in non-DA neurons. As amphetamines release DA from synaptic terminals, it may be that local MA-induced increases in extracellular DA are sufficient to overcome extracellular superoxide dismutase and catalase, which attenuate catecholamine toxicity (Rosenberg, 1988); alternatively, reactive DA or DOPA derivatives such as 6-OHDA, 2,4,5-trihydroxyphenylalanine, or quinones and semiquinones might be released from cells or produced extracellularly (Seiden and Ricaurte, 1987; Olney et al., 1990; Rosenberg et al., 1991). Such mechanisms could play a role in degeneration of neurons receiving DA afferents in striatonigral degeneration (Weiner and Sanchez-Ramos, 1992).

Along with process degeneration and vacuolation, MA induces blebs similar in appearance to those seen during cell death due to chemical hypoxia (Jurkowitz-Alexander et al., 1992) and apoptosis (Wyllie et al., 1980). These structures are generally presumed to arise from deformation of the plasma membrane after cytoskeletal breakdown. The blebs in our experiments ex-

cluded calcein AM, suggesting they are not continuous with the cytoplasm. In contrast to vacuoles, blebs never contained endocytic tracers, implying they did not arise from endocytic organelles. Regardless of whether they occur in MA-treated or control cultures, blebs nearly always exhibit strikingly intense DCF fluorescence, suggesting that hydroperoxides play a role in their formation. Possibly the blebs arise from organelles involved in lipid peroxidation, although a plasma membrane origin cannot be excluded.

Although the present study focuses on MA, similar effects could occur with other weak base drugs toxic to monoamine systems including fenfluramine, *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, methylenedioxymethamphetamine, phencyclidine, and chloroquine, as well as in disorders including Parkinson's disease and striatonigral atrophies (Graham, 1984; Spina and Cohen, 1989; Przedborski et al., 1992; Storey et al., 1992; Weiner and Sanchez-Ramos, 1992). Recently, Beitner-Johnson et al. (1992) found that chronic cocaine decreased neurofilament proteins in midbrain DA regions, suggesting that cocaine may also selectively damage neuronal processes.

*Role of EAAs*

NMDA antagonists protect against MA-induced reduction of striatal DA content and TH activity *in vivo* (Sonsalla et al., 1989, 1991), pointing to a role for EAAs in MA neurotoxicity. However, excitotoxicity per se does not appear to account for MA neurotoxicity. First, we see MA-induced morphological changes in postnatal DA neuron cultures in the presence of glutamate receptor blockade. Second, in contrast to the selective destruction of DA terminals by MA in culture or *in vivo*, striatal injections of excitotoxins such as kainate, ibotenate, and quinolinate destroy cell bodies but spare DA terminals (Olney et al., 1974; Coyle et al., 1978; Schwarcz et al., 1983). Third, excitotoxins are not selective for DA processes either in culture or in the striatum. Rather, our evidence suggests that intracellular DA-dependent oxidative stress may be sufficient to explain the selectivity of MA toxicity for DA terminals.

A DA-dependent model of MA neurotoxicity may well be consistent with the role demonstrated for EAAs *in vivo* (Sonsalla et al., 1989, 1991). For example, the NMDA antagonist MK-801 might protect striatal DA terminals by inhibiting an NMDA-dependent increase in DA release (Bowyer et al., 1991; O'Dell et al., 1992; Weihmuller et al., 1992a,b; Marshall et al., 1993). Tonic NMDA stimulation could increase TH activity, as has been reported with electrical stimulation (Goldstein and Greene, 1987), thereby promoting MA-induced DA release. Oxidative stress could promote neuronal depolarization, leading to NMDA receptor activation and downstream excitotoxicity (Watkins and Collingridge, 1989). Whatever the precise role of EAAs, our observation that MA induces DA-dependent intracellular oxidative stress in the presence of EAA receptor blockade suggests that redistribution of DA from synaptic vesicles to the cytoplasm may be the initiating step in MA neurotoxicity.

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