Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 4865–4869, June 1991
Neurobiology

2,4,5-Trihydroxyphenylalanine in solution forms a non-*N*-methyl-D-aspartate glutamatergic agonist and neurotoxin

(dopamine/3,4-dihydroxyphenylalanine/glutamate/neurotoxicity/hydroxydopamines)

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Communicated by Richard L. Sidman, February 26, 1991

ABSTRACT **We have investigated the pharmacologic and neurotoxic properties of 2,4,5-trihydroxyphenylalanine [topa; the 6-hydroxylated derivative of 3,4-dihydroxyphenylalanine (dopa)] in central neurons. Application of solutions of topa to the chicken eyecup preparation results in glutamatergic responses mediated predominantly by non-*N*-methyl-D-aspartate receptors. Pharmacological activity depends upon oxidation in solution to a new compound. This compound is tentatively identified as topa quinone. Solutions of topa are toxic to cortical neurons in culture, and this toxicity is blocked by the non-*N*-methyl-D-aspartate antagonist 6-cyano-7-nitroquinoxaline-2,3-dione. These results suggest that production or accumulation of topa or its oxidation products might be involved in excitotoxicity, especially in dopaminergic neurons and their projection targets.**

The chemical instability of catecholamines has for many years generated concern regarding their potential for producing neuronal injury and death (1). The mechanism of neurotoxicity usually postulated is oxidative damage by degradation products of catecholamines, including quinoid derivatives (2, 3) and oxygen free radicals (4, 5). Until now, there has been little evidence supporting the possibility that catecholamines or their metabolites might be capable of producing neurotoxicity in the central nervous system by receptor-mediated mechanisms.

Recently a role for dopaminergic systems has been suggested in the neuronal injury and death accompanying cerebral ischemia (6–9), a process mediated in part by glutamate receptor activation (10). In another line of investigation, it has been shown that methamphetamine-induced loss of dopaminergic fibers in the striatum was found to be blocked by the glutamatergic antagonist MK-801 in a stereoselective manner (11). In a third model system, it has been found that the toxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine to substantia nigra may be blocked by several selective *N*-methyl-D-aspartate (NMDA) antagonists (12). These results imply a link between dopaminergic systems and glutamate toxicity and led us to consider the possibility that dopamine, its metabolites, or substances derived from them by normal or abnormal degradation pathways might have glutamatergic properties.

2,4,5-Trihydroxyphenylalanine (topa), a 6-hydroxylated derivative of 3,4-dihydroxyphenylalanine (dopa), has been suggested to be an excitatory agonist in the isolated spinal cord of the frog (13). In a preliminary report, it was demonstrated that application of solutions of topa to rat cortical neurons produced electrophysiological responses as well as neuronal death, which could be blocked by the non-NMDA antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (14). Others demonstrated topa neurotoxicity in the chicken embryo retina, as well as electrophysiological actions of topa in rat hippocampal neurons (15), similar to what we observed in rat cortical neurons (16). In these studies, however, it was not clear whether topa or an oxidation product was the active compound. topa is of particular interest because it can be produced by human melanocyte tyrosinase (17) and has recently been shown to be naturally occurring in the active site of mammalian plasma amine oxidase (18). We have therefore investigated the physiological and pharmacological properties of topa and related substances in the chicken eyecup preparation as well as its toxicity in rat cortical neurons *in vitro*.

METHODS

Electrophysiology. Recordings were obtained from chicken eyecups (5- to 17-day-old chickens) as described (19). For experiments with topa, a freshly made 10 mM stock solution in 1 mM HCl was used. An aliquot of this stock solution was injected into Tyrode's solution and mixed to yield a topa solution of desired concentration. For experiments in which the time course of activation of topa solutions was studied, aliquots of the topa stock solution were injected into physiological saline (PS; 145 mM NaCl/3 mM KCl/1.8 mM $CaCl_2$/1.0 mM $MgCl_2$/8 mM glucose/2.4 mM NaH_2PO_4/0.42 mM Na_2HPO_4, pH 7.2), quickly mixed, and then injected into the preparation after selected times.

Preparation of Dopachrome. A solution of L-dopa (0.5 mg/ml) in sodium phosphate buffer (50 mM, pH 6.8; passed through a Chelex-100 column) was incubated with Ag_2O for 3 min at 0°C, filtered through a 0.22-μm Millex filter, and batch treated with Chelex-100 to remove Ag ions (20).

Tissue Culture. Experiments on cortical neurons used rat embryonic cerebral cortex in dissociated cell culture. Astrocyte-rich cortical cultures were prepared following described methods (21). Approximately 6% of the cells in these cultures were neurons as identified by tetanus immunochemistry (22).

Toxicity Experiments. Coverslip cultures were washed once in PS: 2 ml per 35-mm dish containing five coverslips. The coverslips were then placed in wells containing 0.5 ml of Earle's salt solution/25 mM Hepes, pH 7.3 (ESS/Hepes) with selected concentrations of topa. topa was added from a freshly made 10 mM stock solution in 1 mM HCl. After 30 min, medium was replaced twice with ESS/Hepes and returned to the incubator for 20–24 hr. Experiments were terminated by replacing medium with trypan blue (1:1 dilution with PS), washing once in PS containing 0.01% bovine serum albumin, and fixing with 2.5% glutaraldehyde in PS (5). Neurons were identified by using phase-contrast microscopy

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Abbreviations: topa, 2,4,5-trihydroxyphenylalanine; dopa, 3,4-dihydroxyphenylalanine; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; NMDA, *N*-methyl-D-aspartate; APV, DL-2-amino-5-phosphonovalerate; PS, physiological saline; DTT, dithiothreitol.
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(21). Surviving neurons were identified as those that had excluded trypan blue. Cultures were counted after 24 hr in fixative, at ×125.

CNQX (Tocris Neuramin, Essex, England) was added from a 5 mM stock in 0.3 M NaOH. Experiments were set up at least in duplicate (two coverslip cultures per treatment). Experiments were repeated at least three times. Ten successive fields were counted along a diameter of the coverslip in order to generate cell counts, yielding 500–1000 neurons counted per control coverslip.

RESULTS

Addition of 300 μM topa to the chicken eyecup preparation produced a large response (Fig. 1*A1*), similar in polarity to that produced by either kainate, quisqualate, or NMDA (19). Responses to application of 300 μM topa were inhibited by 5–20 μM CNQX (23) by 79% ± 16% (n = 6) (Fig. 1*A2*). There was no significant difference between the inhibition obtained at 5 μM and at 20 μM, suggesting that these concentrations were at or above the maximal effective concentration for this drug. Responses to application of 300 μM topa were not affected by the NMDA receptor antagonist DL-2-amino-5-phosphonovalerate (APV; 100 μM) (Fig. 1*B1*) (24). No effect of APV on topa-induced responses was seen up to concentrations of 500 μM of the antagonist (n = 4). In contrast, the effect of 500 μM NMDA was completely blocked by 100 μM APV in this preparation (Fig. 1*B2*). The pharmacological properties of topa-induced excitatory responses in the eyecup are similar to what has been observed in whole-cell voltage-clamp recordings in rat cortical neurons (16). We also investigated the dose–response relationship for topa in the eyecup preparation, which is shown in Fig. 1*C*. topa produced measurable responses at concentrations as low as 30 μM and had an EC_{50} of 148 ± 36 μM (mean ± SD; n = 3).

topa rapidly oxidizes in aqueous solution at physiological pH (obvious from the development of orange-red color) (2, 3), and so it was of interest to determine whether the activity of topa-containing solutions was due to topa itself or to an oxidation product. This type of experiment could be easily performed in the eyecup because it is possible to administer drugs to this preparation shortly after preparing solutions. A freshly made solution of 300 μM topa in PS was electrophysiologically monitored for excitatory activity as a function of time. In parallel experiments, we spectrophotometrically measured the time course of the development of visible light absorbance in solutions of 300 μM topa in PS. The results are shown in Fig. 2 *A* and *B*. We found that, immediately after dilution into PS, solutions of topa produced almost no response in the eyecup preparation but developed activity over the next 5 min (Fig. 2*A*). Over a similar time course, solutions of topa in PS developed visible light absorbance, measured in these experiments at 450 nm by using a narrow range interference filter. Therefore the onset of agonist activity of solutions of topa, as well as the increase in potency, seemed to parallel the production of a colored oxidation product.

If the activity of topa solutions was dependent upon the oxidation of topa, then one would expect that the presence of a reducing agent would prevent the development of activity of solutions of topa. Dithiothreitol (DTT) is a reducing agent whose effects on glutamatergic responses in the eyecup preparation have been characterized. DTT potentiates NMDA responses but has no effect on non-NMDA glutamatergic responses (19). We found DTT to be an effective agent for preventing the oxidation of topa at a 10:1 molar ratio, by monitoring the absorbance at 450 nm, thus enabling us to further test whether topa itself or an oxidation product was the active compound. We observed that the addition of 3 mM DTT prevented solutions of 300 μM topa from eliciting a significant response in the eyecup (Fig. 2 *C1* and *C2*). These results together with the previous results shown in Fig. 2 *A* and *B* suggested that the active component of solutions of topa was likely to be one of several possible oxidation products: dopachrome, *ortho*-topa-quinone, or *para*-topa-quinone (2, 3).

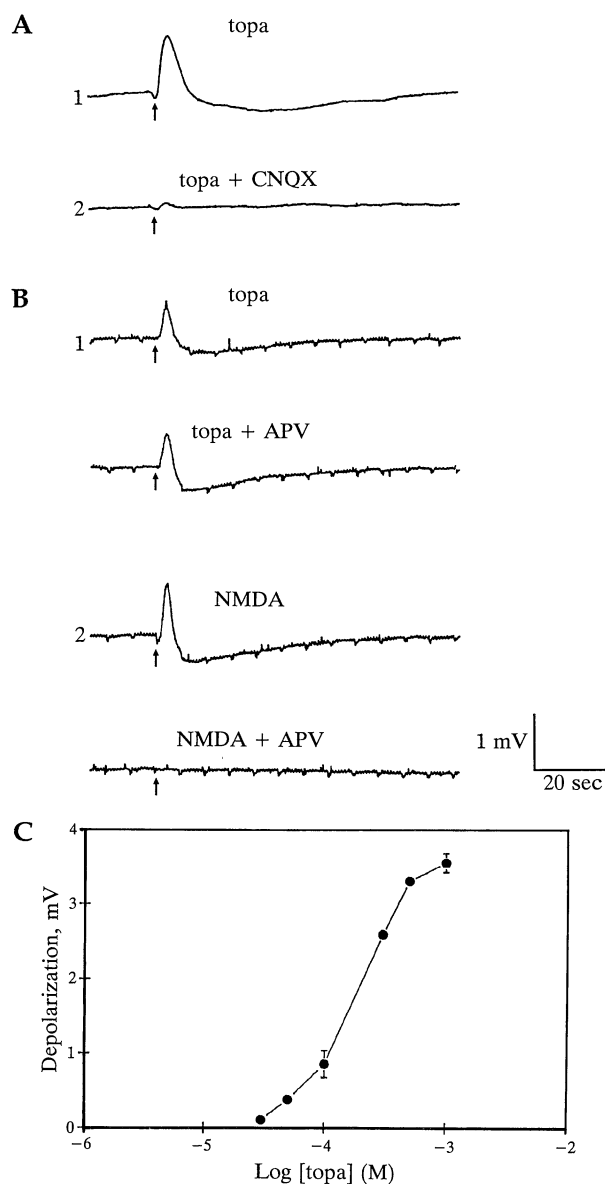

FIG. 1. Pharmacology of topa-induced responses in the chicken eyecup preparation. (*A1*) Response recorded from the chicken eyecup preparation after addition of 300 μM topa via the perfusate. In this and subsequent figures, drugs were applied at the arrow, for 1.5 sec. (*A2*) In the presence of 10 μM CNQX, the response to 300 μM topa was substantially diminished. (*B1*) In another experiment, application of 300 μM topa produced a typical response, which was not blocked by 100 μM APV. (*B2*) The response to 1 mM NMDA was completely blocked in this preparation by 100 μM APV. (*C*) Dose–response relationship of topa. topa at selected concentrations was applied to the eyecup. Peak responses were measured. The means ± SD of three separate applications at each concentration are plotted.

To pursue the identity of the active compound, we were interested in testing the activity of dopachrome, theoretically an oxidation product of topa (2, 3). Dopachrome was synthesized from dopa by silver oxide (Ag_2O) oxidation and had the absorption spectrum shown in Fig. 3*A*. We found dopachrome to be inactive in the chicken eyecup preparation (Fig. 3*A Inset*). When our starting material was topa, an active

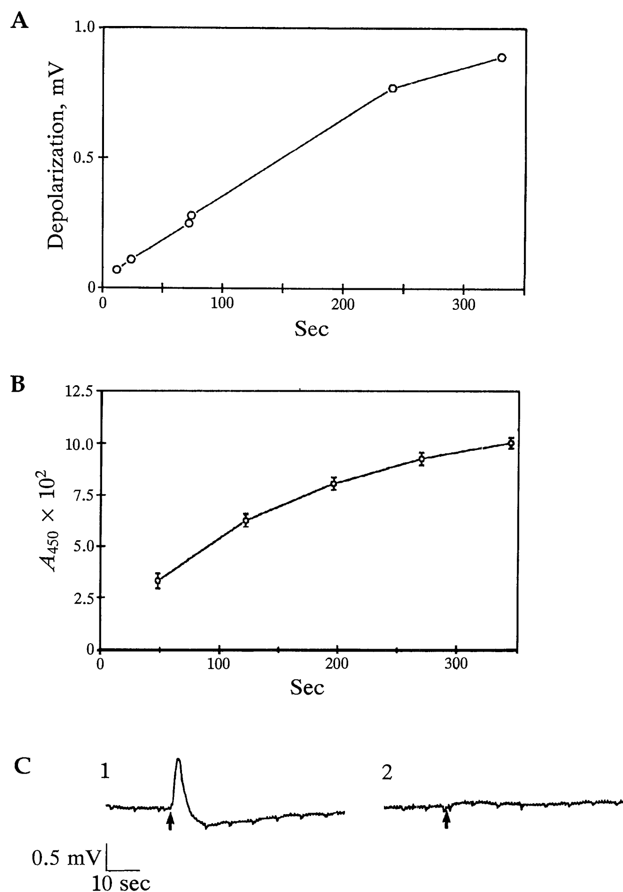

FIG. 2. An oxidation product of topa, not topa, is the active compound. (*A*) A fresh stock solution of 10 mM topa was prepared in 1 mM HCl. At zero time, a 300 μM solution was prepared by injecting an aliquot of the stock solution into PS. At selected times, aliquots of this solution were perfused onto the chicken eyecup, and peak responses were measured and plotted. (*B*) topa was prepared as above, and at selected times, the absorbance at 450 nm of a 200-μl sample was measured by using a multiwell spectrophotometer (Titretek, Flow Laboratories). Measurements were performed on triplicate samples. The means ± SD are plotted. (*C1*) Response recorded from the chicken eyecup preparation after addition of 300 μM topa via the perfusate (at the arrow). (*C2*) A mixture of 300 μM topa and 3 mM dithiothreitol (DTT) failed to elicit a response in the same preparation.

product was obtained (Fig. 3*B Inset*), suggesting that the procedure did not alter the reactivity of the preparation or the ability of the topa product to exert its effect, for example, by virtue of the presence of silver ions. topa allowed to oxidize in solution without the presence of silver oxide had an identical absorption spectrum. Unlike solutions of dopachrome, which are stable for about 30 min on ice and thereafter convert from a deep orange-red to a brown color (suggestive of further oxidation), solutions of the oxidation product of topa (also orange-red) are more stable and persist on ice without color change for hours. As can be seen in Fig. 3, solutions containing dopachrome as well as oxidized topa displayed a broad absorbance peak centered at ≈475 nm. In addition, solutions of dopachrome displayed an absorbance peak in the UV range, at 306 nm, in agreement with the value previously reported (25), in contrast to the UV absorption maximum of 272 nm observed for oxidized topa. Therefore, it appears that oxidation of topa in solution at physiological pH produces a product, distinct from dopachrome, which we assume to be the amino acid topa-quinone (which exists as the tautomers *ortho*- and *para*-topa-quinone). As expected from this identification, we found that the topa oxidation

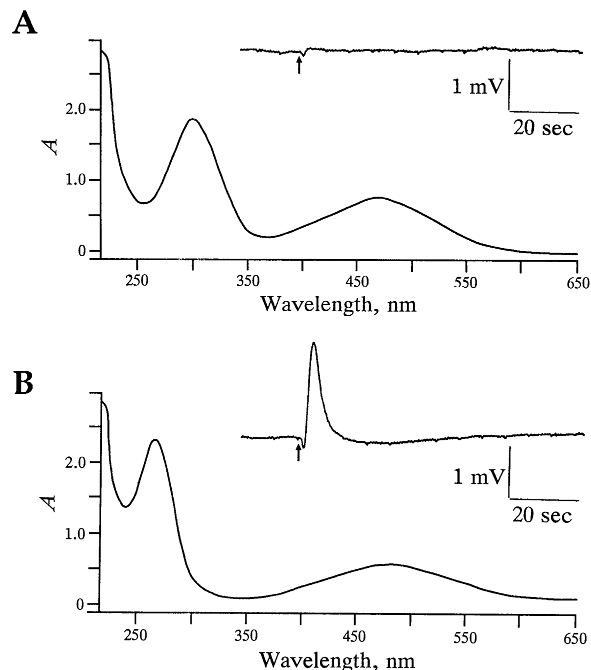

FIG. 3. Dopachrome is distinct from the active compound derived from topa. (*A*) dopa was oxidized in 50 mM sodium phosphate (pH 6.8) by using silver oxide (20); this produced a colored substance whose spectrum is shown, demonstrating an absorption maximum in the UV range at 306 nm and a broad peak in the visible range, as has been described for dopachrome (25). The absorbance spectrum was obtained using an LKB Ultraspec Plus spectrophotometer. (*Inset*) This substance was physiologically inactive in the eyecup preparation. (*B*) topa was oxidized in solution under similar conditions. The absorption spectrum revealed a peak in the UV at 275 nm, distinguishing it from the spectrum obtained of solutions of dopachrome. (*Inset*) Solutions of topa produced in this manner were characteristically active in the eyecup preparation.

product reacted with ninhydrin, whereas dopachrome did not.

Finally, we evaluated the excitotoxic properties of topa in rat cortical cultures. Toxicity experiments were performed on cultures maintained 25–48 days *in vitro* (Fig. 4*A*), using methods that we have used previously (21). Cultures were exposed to topa-containing solutions for 30 min, and toxicity was assayed 20–24 hours later. In eight experiments we found topa to kill cortical neurons with an LD_{50} of 306 ± 111 μM.

To determine the pharmacology of the toxicity of topa, experiments were performed in which cultures were exposed either to topa alone for 30 min or to topa in conjunction with CNQX. CNQX at 37.5 μM was effective in protecting the cultures from the toxic effects of 500 μM topa (Fig. 4*B*). Lower concentrations of CNQX (10 μM) did not consistently produce a significant reduction in the toxicity of 500 μM topa (n = 5). To determine whether the ability of CNQX to block the toxicity of topa was mediated by its action as an antagonist at the NMDA receptor-associated glycine site (26, 27), cultures were exposed to topa plus CNQX in the presence of 1 mM glycine (28). We observed that the presence of glycine did not affect the ability of CNQX to act as an antagonist of the toxic effect of topa on cortical neurons (n = 3). In the experiments shown in Fig. 4*B*, cultures that were exposed to CNQX during topa exposure were also exposed to CNQX following topa, but this postexposure use of CNQX was found not to be necessary to demonstrate the inhibitory effect of CNQX on topa neurotoxicity (n = 2). This result is in contrast to what has been observed regarding the inhibition of quisqualate toxicity by CNQX (29).

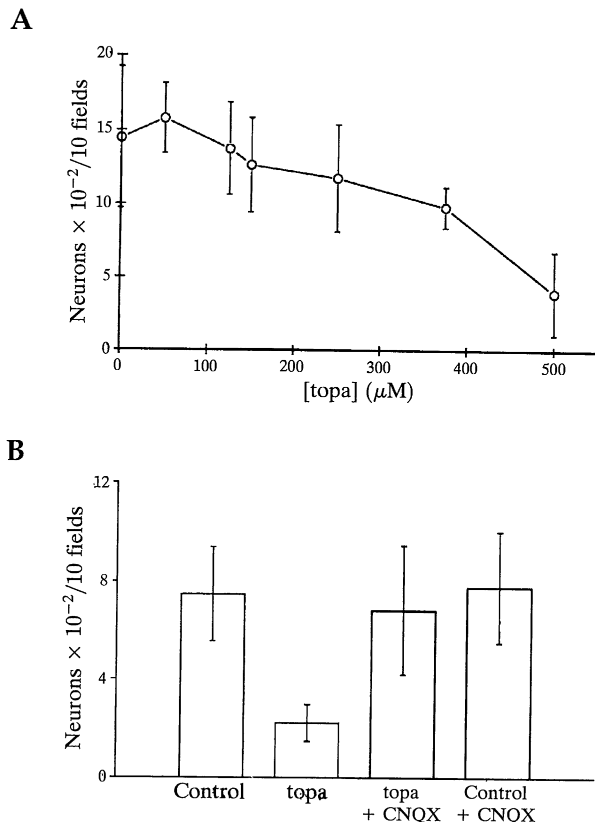

FIG. 4. topa toxicity. (*A*) Dose–response. Cortical cultures were exposed to solutions of topa at selected concentrations for 0.5 hr, washed once, and then returned to the incubator for 20–24 hr. Experiments were terminated by staining with trypan blue, washing, and fixing with 2.5% glutaraldehyde. Neurons in 10 fields were counted in each coverslip, and experiments were performed in duplicate. The results shown represent data pooled from eight such experiments, using five different culture dates. Error bars indicate the SD. (*B*) Pharmacology. Experiments were performed as described above, except that cultures were exposed to vehicle only (Control), 500 μM topa, topa plus 37.5 μM CNQX, or 37.5 μM CNQX (Control + CNQX). CNQX blocked the toxicity of topa, such that there was no significant difference between the control condition and the topa plus CNQX condition. On the other hand, topa alone resulted in a significant ($P < 0.0005$; 2-tailed *t*-test with Bonferroni correction for three comparisons) loss of neurons compared to control cultures. CNQX itself had no effect on neuronal survival. The results shown are pooled from three experiments, each performed in duplicate. Error bars indicate the SD.

Biscoe *et al.* (13) in the frog spinal cord preparation showed that, in addition to topa, L-dopa was also a glutamatergic agonist, although 1/10th as potent. We, however, have not been able to consistently show an effect of L-dopa or DL-dopa at up to 3 mM in the eyecup preparation or in rat cortical neurons at 500 μM (16). We also investigated a series of compounds related to dopa, including dopamine, 6-hydroxydopamine, dihydroxyphenylacetic acid, homovanillic acid, adrenochrome, and homogentisic acid. None of these compounds produced a response in the eyecup preparation at 1 mM.

DISCUSSION

A number of possible mechanisms linking dopaminergic systems in the central nervous system to neurotoxicity have been suggested. These include an effect of dopamine on the high-affinity uptake of glutamate (30), on glucose metabolism (31), on production of free radicals (32), and on potentiation of neuronal responses to excitatory amino acids (33). In addition, the present investigations show that an oxidation product of dopa is a glutamatergic agonist and neurotoxin.

There are two lines of evidence supporting a direct action of topa on cortical neurons, rather than an indirect action, mediated by release of excitatory neurotransmitter(s). (*i*) In a previous study, we showed that perfusion of solutions of topa onto cortical neurons produced immediate membrane responses, observed using whole-cell patch-clamp recording (16). (*ii*) If topa produced its toxic effects by release of excitatory amino acids, in particular glutamate, then one would expect that the toxicity should be blocked by NMDA antagonists, since cortical neurons in culture are vulnerable to both NMDA and non-NMDA receptor-mediated toxicity (10, 29). In fact NMDA antagonists had no effect on topa toxicity (data not shown).

It has been previously recognized that glutamate toxicity in the nervous system may be mediated by endogenous substances other than glutamate and aspartate. For example, quinolinic acid has been identified as a potential NMDA receptor-specific endogenous glutamatergic toxin (34–36), and other possibilities have been suggested as well (10). The present studies suggest that topa quinone may be an endogenous non-NMDA glutamatergic toxin. We have obtained evidence that topa itself (in the reduced form) has no significant pharmacological actions at glutamate receptors (Fig. 2 *A–C*). Since the toxicity is mediated by non-NMDA receptors and since oxidation of topa produces a derivative that is a non-NMDA agonist, we feel it is a reasonable deduction that the toxicity of topa is due to this derivative and not to topa itself.

It has recently been established that topa is found in the primary sequence of mammalian plasma amine oxidase (18), although the mechanism of its production is unknown. A possible endogenous source of topa may be autooxidation of dopa (37, 38). Interestingly, dopa was recently shown to be released by a calcium-dependent mechanism from rat striatal slices after stimulation by nicotine (39). What fraction of dopa may be oxidized to topa, either in the exocytotic vesicle or extracellularly, is unknown. Other sources of endogenous topa may be enzymatic synthesis by melanocyte tyrosinase (17) or other proteins with tyrosinase activity. Whether tyrosinase activity occurs within the melanin-containing nuclei of the brain is controversial (40–42) and is obviously a question of great interest in light of our results. In addition, topa may cross the blood–brain barrier (43), making peripheral production important also.

There are no reports suggesting that topa or topa quinone has been specifically looked for in the normal or abnormal central nervous system, reflecting the fact that their potential importance in human disease has been overlooked. The instability of 6-hydroxy catechols makes the search for topa and its breakdown products technically difficult and may also explain the controversial literature concerning the demonstration of 6-hydroxydopamine in methamphetamine-treated animals (44, 45). The potential significance of finding topa and related compounds in the central nervous system is clear, since it is apparent both from the present as well as previous work that non-NMDA agonists may be neurotoxic without the mediation of NMDA receptors (29, 46–48). In addition, stimulation of non-NMDA receptors might lead to NMDA receptor-mediated toxicity by causing depolarization (and thereby relief of magnesium block) or by release of endogenous excitatory neurotransmitter.

The potency of a toxin is expected to be directly related to the time of exposure, and this has been shown with other non-NMDA agonists (29) and with topa itself (data not shown). There is potentially a continual source of topa in the brain, from catecholaminergic nerve terminals. It is conceivable that prolonged exposure results in a markedly "left-

shifted'' dose–response relationship from the one that we have demonstrated in culture based on a 30-min exposure.

Because of the glutamatergic properties of a dopa oxidation product, catecholaminergic nuclei or their target areas may be at special risk for glutamate receptor-mediated neurotoxicity. The demonstration of the glutamatergic activity of topa provides an incentive to look for this substance and its breakdown products in diseases involving catecholaminergic nuclei, especially in Parkinson disease in which oxidative mechanisms have recently been suggested as playing a pathogenetic role (49). In addition, dopa oxidation products should be looked for in diseases involving brain regions known to be heavily innervated by catecholaminergic axons, such as Huntington disease, schizophrenia, and brain ischemia.

The authors wish to thank Dr. Paul Gallop and Dr. Charles Barlow for helpful discussions and Sara Vasquez for excellent technical assistance. This work was supported by a grant from the Joseph P. Kennedy, Jr. Foundation (P.A.R.), grants from the National Institute of Neurological Disease and Stroke (P.A.R. and R.L.), a grant from the National Institute of Drug Abuse (E.A.), and a Mental Retardation Core Grant to Children's Hospital.

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