Rap1A under similar conditions (*21*). Rac 1, a protein closely related to Rac 2 (92% identity) may also have oxidase regulatory activity. Although Rac 1 mRNA is expressed in a large number of different cell types, Rac 2 mRNA is expressed only in cells of myeloid origin (*16*). In HL-60 cells induced to differentiate with dibutyryl cyclic adenosine monophosphate, expression of Rac 2 mRNA, but not Rac 1 mRNA, increased by seven- to ninefold. This may indicate a specialized action of Rac 2 in neutrophil function or differentiation. Also of interest in this regard are the isoelectric points of the Rac proteins. On the basis of their amino acid sequences, the isoelectric point (pI) of Rac 2 is calculated to be 7.56 and that for Rac 1 is 8.53 (*16*). The four cytosolic oxidase components have been resolved by preparative isoelectric focusing and have approximate pIs of 3.1, 6.0, 7.1, and 9.5. Two of these components (pI 6.0 and 9.5) are $p67_{[phox]}$ and $p47_{[phox]}$, respectively. The pI of 7.1 reported for one of the unidentified factors is similar to that predicted for Rac 2.

The mechanism by which Rac 2 acts to enhance activity of the NADPH oxidase and its exact function in the assembly and translocation of the other cytosolic components to form an active complex at the membrane should be amenable to investigation. Additionally, the availability of a cell-free assay for the activity of this member of the Ras superfamily will enhance our ability to determine regulatory features of the low molecular weight GTP-binding proteins in general.

Note added in proof: It has recently been reported that Rac 1 also has oxidase regulatory capability (*22*).

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NMDA Antagonist Neurotoxicity: Mechanism and Prevention

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Antagonists of the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptor, including phencyclidine (PCP) and ketamine, protect against brain damage in neurological disorders such as stroke. However, these agents have psychotomimetic properties in humans and morphologically damage neurons in the cerebral cortex of rats. It is now shown that the morphological damage can be prevented by certain anticholinergic drugs or by diazepam and barbiturates, which act at the γ-aminobutyric acid (GABA) receptor-channel complex and are known to suppress the psychotomimetic symptoms caused by ketamine. Thus, it may be possible to prevent the unwanted side effects of NMDA antagonists, thereby enhancing their utility as neuroprotective drugs.

ANTAGONISTS OF THE NMDA SUBtype of glutamate receptor are potentially useful for preventing neuronal degeneration in neurological disorders such as stroke (*1*). However, treatment of adult rats with noncompetitive (phencyclidine, MK-801, tiletamine, ketamine) or competitive [D-2-amino-5-phosphonopentanoate (D-AP5)] NMDA antagonists causes neurotoxic side effects consisting of pathomorphological changes in neurons of the cingulate and retrosplenial cerebral cortices (*2, 3*). After low doses these changes may be reversible, but higher doses can cause irreversible neuronal necrosis (*4*). Therefore, it has been questioned whether NMDA antagonist therapy can be applied without incurring serious side effects. However, we now report that certain anticholinergic or GABAergic agents protect cerebrocortical neurons against the adverse side effects of NMDA antagonists.

The neurotoxic action of MK-801 in the adult rat cingulate cortex is potentiated by pretreatment with the cholinergic agonist pilocarpine (*5*). This potentiating effect was abolished by coadministration of scopola-

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mine, a cholinergic muscarinic antagonist. These results suggested that activation of muscarinic receptors might be involved in the process by which MK-801 causes neurotoxic side effects. To explore this possibility, we administered scopolamine intraperitoneally (ip) in various doses (0.01 to 5 mg per kilogram of body weight) 10 min after a subcutaneous (sc) dose of MK-801 (0.4 mg/kg) that reliably causes neurotoxic side effects in 100% of treated rats. We examined the brains after 4 hours and found that scopolamine completely prevented MK-801 neurotoxicity at doses ≥0.25 mg/kg. Dose-response studies revealed that the ED_{50} (dose of scopolamine that prevented MK-801 neurotoxicity in 50% of treated animals) was 0.13 mg/kg. Additional anticholinergic compounds were also effective (Table 1), with an order of potencies correlating with their binding affinities for M1 muscarinic receptors (*6*).

To determine whether anticholinergic agents can protect against the neurotoxic effects of noncompetitive NMDA antagonists other than MK-801, we treated six rats with a neurotoxic dose (5 mg/kg sc) of PCP and six rats with this dose of PCP plus scopolamine (0.5 mg/kg ip) and killed the animals 4 hours later. All of the rats treated with PCP alone had conspicuous vacuolar changes in cingulate and retrosplenial cortical neurons, whereas none of the rats treated with PCP plus scopolamine had such changes.

In order for NMDA antagonists to be optimally useful as neuroprotective agents in conditions such as stroke, it may be necessary to use relatively large doses. Therefore, we conducted experiments to determine whether the dose of anticholinergic agent required to prevent neurotoxic side effects of a low dose of NMDA antagonist would also protect against a high dose. It required 0.25 mg/kg ip scopolamine to prevent the neurotoxic side effects in 100% of treated rats (n = 6) after a relatively low dose of MK-801 (0.4 mg/kg sc); therefore, we treated adult rats with this dose of scopolamine plus a high dose of MK-801 (5 mg/kg sc) and found that it prevented the neurotoxic side effect in all animals (n = 6), whereas all controls (n = 6) that received the high dose of MK-801 by itself had a severe vacuole reaction in cingulate-retrosplenial cortical neurons.

Using a chick embryo retina assay, we conducted experiments to determine whether anticholinergic drugs interfere with the neuroprotective actions of NMDA antagonists. We have shown that the neurotoxic action of NMDA (120 μM) in the chick retina is prevented by adding 200 nM MK-

Table 1. Prevention of MK-801 neurotoxicity by anticholinergics and barbiturates. A total of 376 adult Sprague-Dawley rats were treated with MK-801 (0.4 mg/kg sc); controls received only MK-801, and experimentals received an ip injection of an anticholinergic or barbiturate drug in various doses 10 min after MK-801 injection. We tested each experimental drug at four or more doses, using at least four rats per dose. Ten or more rats that received only MK-801 were included as internal controls for each experiment pertaining to a given drug. Four hours after treatment, all rats were killed for histological evaluation of their brains (*2*). Two experienced histopathologists who were blind to the treatment conditions evaluated histological sections from the cingulate and retrosplenial cortices of each animal for evidence of intracytoplasmic vacuole formation. All of the control rats (n = 164) were scored strongly positive for vacuole formation. An ED_{50} was calculated by linear regression analysis with the 25th and 75th percentiles (parentheses) defining the confidence limits. For each test compound, we identified an ED_0 and ED_{100}, doses that protected 0 and 100% of treated rats, respectively, and found that the latter consistently was approximately two to three times higher than the former.

Test compound	ED_{50} (mg/kg ip)	Confidence limits
Anticholinergics		
Scopolamine	0.13	(0.08–0.18)
Benztropine	1.29	(0.92–1.65)
Trihexyphenidyl	2.41	(1.26–3.57)
Atropine	2.67	(1.67–3.67)
Biperiden	3.70	(1.40–6.00)
Procyclidine	4.52	(2.25–6.75)
Benactyzine	7.53	(6.25–8.75)
Diphenhydramine	17.54	(13.75–21.25)
Barbiturates		
Pentobarbital	13.07	(9.48–16.65)
Secobarbital	15.28	(10.91–19.66)
Thiopental	27.38	(16.13–38.62)
Thiamylal	29.86	(20.59–39.14)

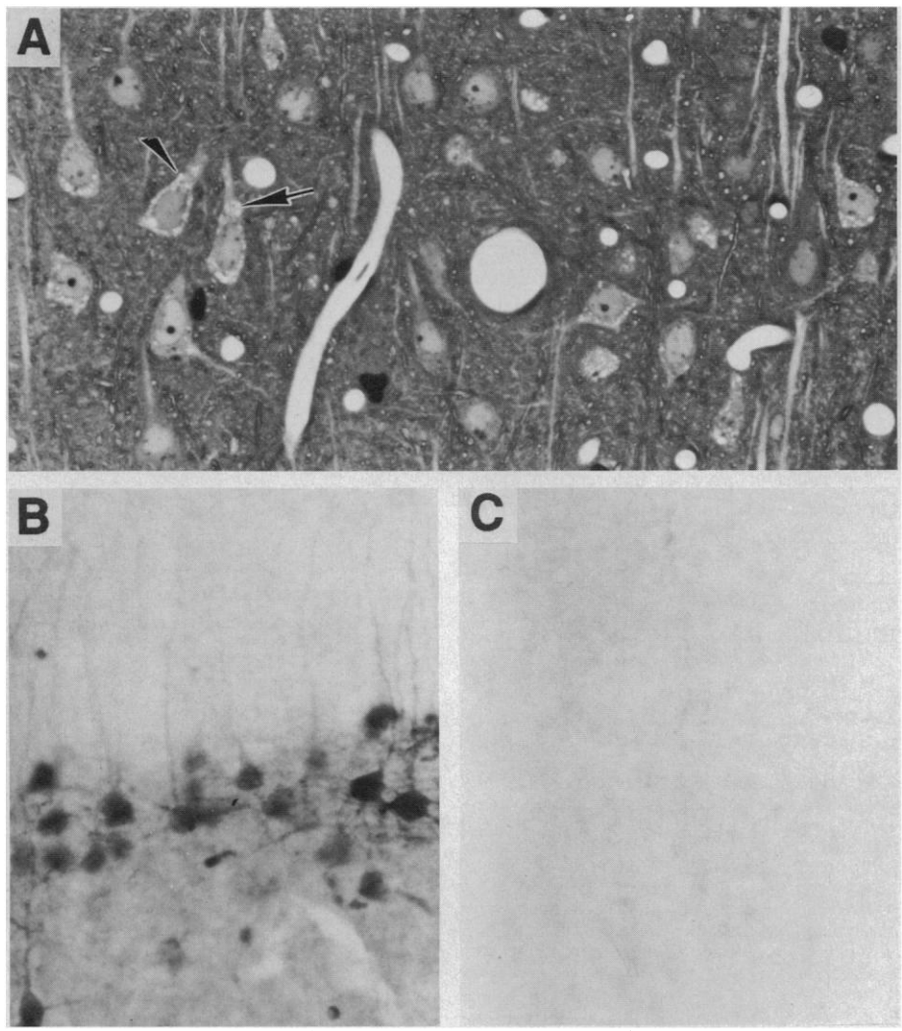

Fig. 1. (**A**) Neurons in layer III of the posterior cingulate cortex of a rat 4 hours after intravenous administration of CPP (50 mg/kg). Arrows indicate vacuoles. Histological methods have been described (*2*). By electron microscopy (not shown), the vacuolar reaction and mitochondrial changes were identical to those induced by MK-801 and PCP. (**B**) Neurons in layer III of the posterior cingulate cortex displaying selective immunoreactive staining to an antibody of HSP70 (*10, 11*). This rat was treated with MK-801 (0.4 mg/kg sc) 24 hours earlier. (**C**) Light microscopic section from the same posterior cingulate cortical area as in (B), but from an animal that received MK-801 (0.4 mg/kg sc) followed 10 min later by scopolamine (0.5 mg/kg ip). [(A) ×320; (B and C) ×280)].

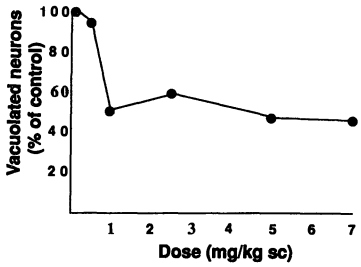

Fig. 2. Effects of diazepam on vacuole formation. Adult Sprague-Dawley rats were treated with MK-801 (0.4 mg/kg sc); controls (n = 18) received only MK-801 and experimentals (n = 36) received an ip injection of diazepam 10 min after MK-801 injection. Four hours after treatment, all rats were killed and their brains prepared for histological evaluation as described (*2*). We quantitated the neurotoxic response as follows: A histological section cut through the posterior cingulate cortex at a specific stereotaxic coordinate (5.3 mm posterior to bregma) (*18*) was selected from each brain, and the number of cingulate neurons containing vacuoles was counted. The mean number of vacuolated neurons for each experimental group was divided by the control mean to permit expressing the severity of the reaction in experimental brains as a percentage of controls.

801 to the incubation medium. Therefore, we incubated the chick retina in medium containing NMDA (120 μM) and MK-801 (200 nM) and added scopolamine in various concentrations from 10 to 50 μM. Scopolamine did not interfere with the ability of MK-801 to prevent NMDA neurotoxicity. Thus, a tissue concentration of scopolamine 250 times higher than that of MK-801 does not interfere with the neuroprotective properties of MK-801, whereas a dose of scopolamine only 1/20 as high as the MK-801 dose prevented the neurotoxic side effects of MK-801 in rodent cortex.

The competitive NMDA antagonist D-AP5, when injected into the cingulate cortex, causes a neurotoxic reaction identical to that caused by systemic MK-801 or PCP (*3*). In the present study, we extended these experiments to include systemic administration of 3-[(±)-2-carboxypiperazin-4-yl]-propyl-1-phosphonic acid (CPP), a competitive NMDA antagonist that is more potent than D-AP5. Intravenous (iv) administration of CPP in a dose of 50 mg/kg caused a vacuole reaction in six of six rats (Fig. 1A) that was identical to the reaction in six of six positive controls that received MK-801 (0.4 mg/kg sc) and was not present in six of six controls treated with normal saline. Administration of scopolamine (0.5 mg/kg ip) 10 min after CPP (50 mg/kg iv) prevented this reaction in six of six rats.

The psychotomimetic effects of PCP include hallucinations, agitation, and disorientation. Ketamine, a PCP receptor ligand, when used in humans as an anesthetic, causes similar psychotomimetic effects termed an "emergence" reaction (*7*). Because diazepam reduces the severity of the psychotomimetic side effects of ketamine and is used widely in human anesthesia for this purpose (*8*), we studied the effects of diazepam on NMDA antagonist neurotoxicity. Because barbiturates, such as diazepam, act at $GABA_A$ receptors, we also tested several barbiturates. At a dose of 1 mg/kg ip, diazepam provided up to 50% protection; this effect could not be exceeded by increasing the dose sevenfold (Fig. 2). However, each of four barbiturates completely protected against NMDA antagonist neurotoxicity with a steep dose-response curve (Table 1). The protection conferred by barbiturates cannot be attributed to general anesthesia properties because the nonbarbiturate anesthetic halothane did not suppress NMDA antagonist neurotoxicity (*9*).

Although the neurotoxic reaction appears to be confined to specific neurons within the cingulate and retrosplenial cortices, the pathological action of NMDA antagonists may not be limited to these neuronal populations. These agents induce heat shock protein (HSP) not only in the specific neurons that undergo pathomorphological changes but also in several other types of forebrain neurons (*10, 11*). This HSP response is blocked by scopolamine (Fig. 1, B and C) or GABAergic agents. Thus, the HSP response may be triggered by the same toxic mechanism that causes vacuolization of cerebrocortical neurons and may serve as an alternate marker of neuronal susceptibility to this type of injury.

Our evidence that either competitive or noncompetitive NMDA antagonists cause identical neurotoxic side effects, and either muscarinic cholinergic antagonists or GABAergic agents block these side effects, suggests that the effect is triggered by suppression of NMDA receptor function but also involves muscarinic and GABAergic receptors. Figure 3 schematically depicts a set of neural connections that can explain these findings.

The difference in efficacy between diazepam and barbiturates may relate to their different modes of action at the GABA receptor. Barbiturates act directly to open the chloride channel, even in the absence of GABA (*12*), whereas diazepam acts only to potentiate the action of GABA (*13*). Because blockade of the NMDA receptor (Fig. 3) would result in cessation of GABA release, there would be very little GABA in the synaptic cleft for diazepam to potentiate. Therefore, consistent with our findings, diazepam should have only a partial effect in contrast to barbiturates which, like anticholinergics, should provide complete protection against the pathomorphological effects of the NMDA antagonist.

Typically, diazepam has been found to be partially but not completely effective in eliminating emergence symptoms associated with ketamine anesthesia (*8*). We found no studies pertaining to the use of anticholinergics for this purpose, and only a single study (*14*) pertaining to barbiturates. In this study, a single dose of thiopental (2 to 3 mg/kg) provided more complete protection against ketamine emergence reactions than has been reported for diazepam (*8, 14*).

Because GABAergic agents can prevent both the morphopathological and psychopathological side effects of NMDA antagonists, a common mechanism may underlie both effects (*15*). If so, it is possible that either GABAergic or anticholinergic drugs may provide a simple and safe (*16*) method of preventing both psychotomimetic and neurotoxic side effects of NMDA antagonists without interfering (*17*) with the neuroprotective properties of these compounds.

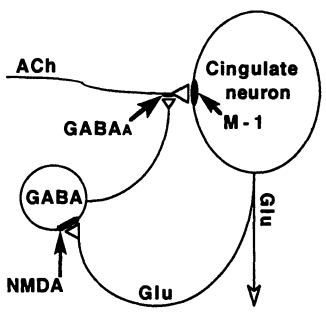

Fig. 3. A proposed wiring diagram to explain NMDA antagonist neurotoxicity. The cingulate neuron is glutamatergic with an axon collateral feeding back to an NMDA receptor on a GABAergic neuron to maintain tonic inhibitory control over the release of acetylcholine (ACh) at an M1 muscarinic receptor on the cingulate neuron's surface. Blockade of the NMDA receptor abolishes the inhibitory control over ACh release and subjects the cingulate neuron to a state of persistent cholinergic hyperstimulation, which we propose is the proximate cause of the pathomorphological and HSP reactions in the cingulate neuron. These reactions can be prevented either by restoring GABAergic inhibitory tone (for example, with barbiturates) or by blocking M1 cholinergic receptors. Diazepam is only partially effective in restoring GABAergic tone because its efficacy depends on the presence of GABA at the $GABA_A$ receptor and the NMDA antagonist has inactivated the GABAergic neuron, thereby abolishing its release of GABA.

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15. The proposal that the pathomorphological and psychotomimetic side effects of NMDA antagonists may be causally linked rests on the reasonable assumption that reversible injury confined to cingulate-retrosplenial neurons might produce a temporary derangement in psychological functions mediated by any or all components of an extensive neural network with which these neurons communicate.
16. Behavioral side effects of competitive and noncompetitive NMDA antagonists are similar and consist of ataxia and hyperactivity at low to moderate doses, with a progressive increase in muscle tone at higher doses that causes the animals to lie on their sides with partially flexed limbs held in a rigid posture. Anticholinergic drugs were well tolerated; in fact, they tended to relieve these symptoms, especially the muscular rigidity. Treatment with an NMDA antagonist plus a barbiturate was also well tolerated. Consistent with the barbiturate effect alone, the animals appeared heavily sedated, but there was no apparent potentiation by the barbiturate of the NMDA antagonist's effects or vice versa, and respiratory function was not compromised.
17. Certain barbiturates, especially thiamylal, effectively block both NMDA and non-NMDA subtypes of glutamate receptor and can prevent ischemic neuronal degeneration [J. Olney *et al.*, *Neurosci. Lett.* **68**, 29 (1986); J. Olney, in *Excitatory Amino Acids in Health and Disease*, D. Lodge, Ed. (Wiley, London, 1988), pp. 337–352]. Thus, barbiturates are neuroprotective in a dual sense. They protect against ischemic neuronal degeneration in many brain regions by blocking glutamate (including NMDA) receptors, while preventing NMDA antagonist neurotoxicity in the cingulate cortex by exerting GABAmimetic activity that is stronger than their NMDA antagonist activity.
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Functional Contribution of Neuronal AChR Subunits Revealed by Antisense Oligonucleotides

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Although multiple related genes encoding nicotinic acetylcholine receptor (AChR) subunits have been identified, how each of these subunits contributes to AChRs in neurons is not known. Sympathetic neurons express four classes of AChR channels and six AChR subunit genes (α3, α4, α5, α7, β2, and β4). The contribution of individual subunits to AChR channel subtypes in these neurons was examined by selective deletion with antisense oligonucleotides. An α3 antisense oligonucleotide decreased the number and altered the properties of the normally expressed ACh-activated channels. The remaining AChR channels have distinct biophysical and pharmacological properties that indicate an important functional contribution of the α7 subunit.

LIGAND-GATED ION CHANNELS ARE multimeric protein complexes with component subunits encoded by homologous genes (*1*). The neuronal nicotinic AChRs that mediate neurotransmission at many central and peripheral synapses comprise two subunit types (α and β) encoded by at least 11 related genes (*2*). Expression of particular combinations of the subunit RNAs in oocytes yields biophysically and pharmacologically distinct channels (*3*), reminiscent of the multiple AChR channel types detected in embryonic sympathetic neurons (*4*). However, neither the identity nor the functional contribution of the individual α and β subunits in native AChR channel subtypes is known.

To determine which AChR subunit genes are expressed by sympathetic neurons, we used the polymerase chain reaction (PCR) (*5*). Primers to the nonconserved regions of the α and β subunit genes were chosen to amplify specific cDNA target sequences (Fig. 1A). Appropriate-sized products were generated in the α3, α4, α5, α7, and β reactions, but not in the α2 reaction (Fig. 1B) (*6*). Restriction enzyme digestion of the amplified products confirmed their identity. RNA blots of polyadenylated [poly(A)$^+$] RNA from sympathetic ganglia under stringent hybridization and washing conditions revealed hybridization to α3, α4, α5, α7, β2 (n-α), and β4 (n-α3) probes but not to the α2 probe (Fig. 1C) (*7*). AChR gene expression ranged from undetectable (α2) and low (α4, α5, β2) to relatively high (α3, α7, β4).

Because sympathetic neurons express multiple types of α and β subunit genes, the AChR channel subtypes observed (*4*) might result from different subunit combinations. To determine the role of individual AChR subunits in sympathetic neurons, we examined changes in the properties of AChR channels after functional deletion of a particular subunit with antisense oligonucleotides.

The contribution of preexistent surface AChRs was removed by treatment with bromoacetylcholine (BAC), which covalently binds to and inactivates AChRs (*8*). BAC-treated neurons regain ACh sensitivity through synthesis of new AChRs (*9*). ACh sensitivity was <15% of control at 3 hours and completely restored within 48 hours of BAC treatment (*9*). Sympathetic neurons were incubated with oligonucleotide immediately after BAC treatment, and the ACh-activated single channel currents were measured after 48 hours (*10*). Analysis of the number of channel openings and the averaged single channel current from all patches in α3 antisense–oligonucleotide treated neurons indicates a >90% decrease from the control levels of AChR channel activity typically evoked by 2.5 μM ACh (Fig. 2) (*11*). Although the number of AChR channel openings under these conditions was too low for slope conductance determinations, the residual activity included openings at amplitudes consistent with the characterized AChR subtypes (designated S, M, L, and XL) (*4*). An α4 antisense (Fig. 2) as well as several control oligonucleotides (*10*) did not affect AChR channel opening frequency, mean current, conductance, or desensitization kinetics. In view of these results and the lack of effect on other ligand or voltage-gated channels (*10*), it seems likely that α3 antisense reduces AChR channel activity by a specific functional deletion of the α3 subunit.

The observed decrease in AChR channel activity gated by 2.5 μM ACh is apparently due to both an increase in the ACh concentration required for channel activation and a decrease in the number of functional AChRs (Figs. 3 and 4). In patches from control

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