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Neuroprotective Effects of Glutamate Antagonists and Extracellular Acidity

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Glutamate antagonists protect neurons from hypoxic injury both in vivo and in vitro, but in vitro studies have not been done under the acidic conditions typical of hypoxia-ischemia in vivo. Consistent with glutamate receptor antagonism, extracellular acidity reduced neuronal death in murine cortical cultures that were deprived of oxygen and glucose. Under these acid conditions, *N*-methyl-D-aspartate and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate–kainate antagonists further reduced neuronal death, such that some neurons tolerated prolonged oxygen and glucose deprivation almost as well as did astrocytes. Neuroprotection induced by this combination exceeded that induced by glutamate antagonists alone, suggesting that extracellular acidity has beneficial effects beyond the attenuation of ionotropic glutamate receptor activation.

Glutamate receptor antagonists reduce the neuronal death induced by hypoxia-ischemia in vivo, most likely by reducing excitotoxic injury (*1*). Glutamate antagonists also reduce neuronal death in vitro after hypoxic insults (*2*). However, most in vitro studies have been performed at physiological extracellular pH (7.2 to 7.4), whereas the pH of hypoxic-ischemic brain in vivo is usually more acidic (pH 6.4 to 6.9).

Extracellular acidity itself may contribute to brain injury (*3*), perhaps by damaging astrocytes and promoting tissue necrosis (*4*), but it may also improve neuronal survival by reducing excitotoxicity (*5–8*). Glutamate receptor–mediated whole cell currents in hippocampal neurons, especially currents mediated by *N*-methyl-D-aspartate (NMDA) receptors, are attenuated at acidic extracellular pH (pH 6.6) (*5*). Cortical (*6*) and cerebellar neurons (*7*) are similarly affected, and moderate acidity reduces rapidly triggered glutamate neurotoxicity and hypoxic neuronal injury in cell cultures and hippocampal slices (*6, 8*).

We examined the neuroprotective efficacy of glutamate antagonists in vitro, under conditions of extracellular acidity relevant to hypoxia-ischemia in vivo (*9*). Loss of parenchymal neuroprotective efficacy might occur if such acidity eliminated the contribution of glutamate receptors to excitotoxic injury. In that case, neuroprotective effects documented in vivo might be explained by other actions, for example, effects on blood flow (*10*).

Mixed neuronal and glial cell cultures, and astrocyte cell cultures, derived from fetal mice on days 15 through 17 of embryonic development were cultured for 14 to 16 days (*11*). We deprived neuronal-glial cultures of oxygen and glucose by exchanging the bathing medium within a hypoxia chamber (<0.3% O_2) (*12*).

Oxygen and glucose deprivation at pH 7.4 for longer than 40 min induced gross neuronal degeneration within 24 hours, associated with lactate dehydrogenase (LDH) efflux into the bathing medium (*13*). The glial layer remained morphologically intact and excluded trypan blue dye.

Table 1. Enhancement of neuroprotection by MK-801 in the presence of extracellular acidity. Sister cultures were deprived of oxygen and glucose for 120 min, alone (CTRL) or in the presence of 10 μM MK-801 or 10 μM CNQX, at pH 7.4 or 6.4. Values represent the mean LDH ± SEM (n = 4), measured 24 hours after exposure, scaled to the mean LDH found in control injury at pH 7.4 (= 100). Significant difference ($P < 0.05$) from control injury, pH 7.4 (*), or from control injury, pH 6.4 (†), was determined by analysis of variance and Student-Neuman-Keuls' test.

System	LDH	Significance
	pH = 7.4	
CTRL	100 ± 11	
10 μM MK-801	61 ± 5	*
	pH = 6.4	
CTRL	63 ± 2	*
10 μM MK-801	9 ± 4	*†
10 μM CNQX	41 ± 9	*

Complete neuronal degeneration was induced by oxygen-glucose deprivation for 70 to 100 min (Fig. 1). In astrocyte cultures, glial damage and LDH efflux did not occur until deprivation approached 5 hours. Lowering the pH of the medium to 6.4 markedly attenuated the neuronal death induced by deprivation for 40 to 80 min; however, this protective effect diminished with deprivation for longer periods and was largely absent after deprivation for 100 min (Fig. 1).

We examined the effect of glutamate receptor antagonists at pH 6.4, using periods of oxygen-glucose deprivation sufficient to override the protective effect of acidity alone. We found that the noncompetitive NMDA antagonist MK-801 (*14*) reduced neuronal injury (Table 1 and Fig. 2), whereas the α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionate (AMPA)–kainate receptor antagonist 6-cyano-7-nitro-quinoxaline-2,3-dione (CNQX) (*15*) alone did not have a significant neuroprotective effect (Table 1). The noncompetitive NMDA antagonist dextrorphan (*16*) and the competitive NMDA antagonist CGS-19755

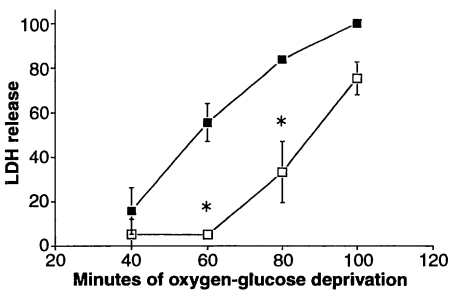

Fig. 1. Extracellular acidity reduces neuronal injury induced by oxygen-glucose deprivation. Sister cultures (derived from the same plating) were exposed to medium lacking oxygen and glucose for the periods indicated, at pH 7.4 (filled squares) or pH 6.4 (open squares). Values represent the mean LDH ± SEM (n = 4), scaled to the near complete neuronal degeneration (assessed by phase-contrast microscopy and trypan blue staining) induced by 100 min of oxygen-glucose deprivation at pH 7.4 (= 100). An asterisk indicates a significant difference from the same duration of deprivation at pH 7.4 ($P < 0.05$) by analysis of variance and Student-Neuman-Keuls' test. Points without error bars indicate SEM less than the width of the plotted point, and background LDH measured in cultures exposed to sham wash has been subtracted from all values.

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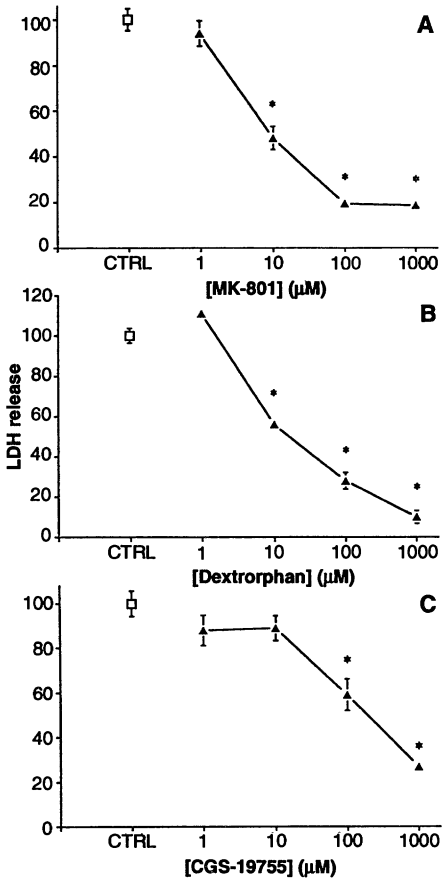

Fig. 2. Neuroprotection provided by NMDA antagonists at acid pH. (**A**) Sister cultures were deprived of oxygen and glucose for 120 min at pH 6.4, alone (CTRL) or in the presence of MK-801. Values represent the mean LDH ± SEM (n = 4) scaled to the mean LDH found in control injury (= 100). An asterisk indicates a significant difference ($P < 0.05$) from the control, determined by a two-tailed t test with Bonferroni correction. (**B**) Same as (A) but with dextrorphan. (**C**) Same as (A) but with CGS-19755.

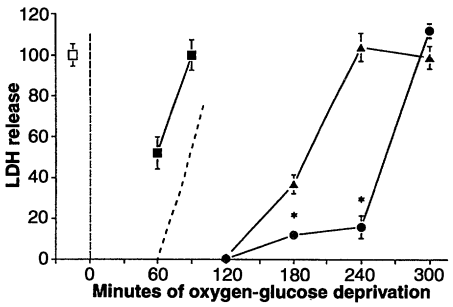

Fig. 3. Extracellular acidity further reduces neuronal death in the presence of NMDA and AMPA-kainate receptor blockade. Sister cultures were exposed to medium lacking oxygen and glucose for the times indicated, alone (control, pH 7.4, filled squares) in the presence of 30 μM MK-801, 300 μM 7-chlorokynurenate and 100 μM CNQX at pH 7.4 (filled triangles), or in the presence of the same antagonists at pH 6.4 (filled circles). Values represent the mean LDH ± SEM (n = 4), scaled to the mean value (= 100) measured after exposure for 24 hours to 500 μM NMDA (open square, which corresponds to near-complete neuronal degeneration by morpholcgical criteria). The dashed line indicates the dose-response curve seen in the presence of 10 μM MK-801 at pH 6.4 (Fig. 1). An asterisk indicates a significant difference ($P < 0.05$) between glutamate antagonist conditions at pH 7.4 and 6.4, determined by analysis of variance and Student-Neuman-Keuls' test.

(*17*) also produced neuroprotection (Fig. 2). Consistent with prior observations at pH 7.4 (*18*), 10 or 100 μM CNQX did reduce neuronal death at pH 6.4 when combined with MK-801 (Table 2); in fact, neuroprotection was greater at pH 6.4 than at pH 7.4 (Table 2).

With blockade of both NMDA and AMPA-kainate receptors, longer oxygen-glucose deprivation was required at pH 6.4 than at pH 7.4 to produce the same amount of neuronal death (Fig. 3). We added 7-chlorokynurenate, an antagonist of the glycine site on NMDA receptors (*19*), to MK-801 and CNQX to ensure antagonism of NMDA receptors. When acidity was combined with blockade of both NMDA and AMPA-kainate receptors, most cortical neurons survived oxygen-glucose deprivation for up to 240 min. Only when deprivation was extended to 300 min did widespread neuronal injury occur (Fig. 3). At this point, LDH release probably reflected damage to both neurons and glia, as some glial cells took up trypan blue dye.

We conclude that glutamate antagonists retain neuroprotective efficacy in cortical cell cultures exposed to oxygen-glucose deprivation under conditions of extracellular acidity that accompany hypoxia-ischemia in vivo. The neuroprotective effects of NMDA antagonists at pH 6.4 suggest that some cytotoxic activation of neuronal NMDA receptors occurs at that pH, an idea consistent with the results of electrophysiological studies (*6*). Dextrorphan and MK-801 were less potent neuroprotective agents at pH 6.4 (Fig. 3) than at pH 7.4 (*20*). Contributing to this loss of potency may be the increased severity of the oxygen-glucose deprivation, alterations in NMDA receptor–complex affinity for antagonist drugs at acidic pH, or protective effects associated with the nonspecific blockade of cation channels at high drug concentrations (*21*).

At both pH 7.4 (*9*) and pH 6.4, AMPA-kainate and NMDA receptor antagonists together reduced neuronal death to a greater degree than either receptor antagonist alone. At pH 6.4, this combined antagonism reduced neuronal death below that achieved with maximal glutamate receptor blockade at normal pH, to the point that neurons became almost as injury-resistant as glia. The additional protection conferred by acidity suggests that it may have neuroprotective actions independent of excitotoxicity reduction (*22*). A protective effect of acidity has been observed on cardiac, renal, and liver cells exposed to hypoxia (*23*), possibly mediated by alterations in cellular metabolism or in cellular Ca^{2+} homeostasis. In muscle, acidity inhibits Ca^{2+} influx through the slow inward current (*24*) and Na^{+}-Ca^{2+} exchange (*25*), as well as Ca^{2+} release by sarcoplasmic reticulum (*26*). Furthermore, acidity may inhibit the binding of Ca^{2+} to key protein or lipid sites responsible for triggering cytotoxic events (*27*).

The idea that extracellular acidity could reduce NMDA receptor–mediated excitotoxicity was proposed to help explain the observed lack of benefit of NMDA antagonists in global hypoxic-ischemic injury (*28*). However, our observations suggest that NMDA receptors do contribute to neuronal death under acidic conditions and thus that NMDA antagonists may retain therapeutic value at low pH. Perhaps NMDA receptor–mediated neurotoxicity in global ischemia is masked by other injury mechanisms. Verification of this idea will

Table 2. Addition of an AMPA-kainate receptor antagonist enhances NMDA antagonist neuroprotection under acidic conditions. Sister cultures were deprived of oxygen and glucose for 150 min at pH 7.4 or 6.4, alone (CTRL), in the presence of 10 μM MK-801, or in the presence of 10 μM MK-801 together with 10 or 100 μM CNQX. Values represent the mean of summed LDH measurements ± SEM (n = 4), immediately before removal of medium lacking oxygen and glucose and 24 hours later, scaled to the mean LDH found in controls at pH 7.4 (= 100). An asterisk indicates a significant difference ($P < 0.05$) from the control at the same pH by analysis of variance and Student-Neuman-Keuls' test. LDH release associated with MK-801 + 10 or 100 μM CNQX at pH 6.4 was significantly different ($P < 0.05$) from that associated with MK-801 + 100 μM CNQX at pH 7.4 (†) and that associated with MK-801 alone at pH 6.4 (‡). The duration of the deprivation was sufficient to destroy most of the neuronal population even in the presence of MK-801 at pH 6.4.

System	LDH	Significance
	pH = 7.4	
CTRL	100 ± 6	
10 μM MK-801, 100 μM CNQX	77 ± 5	
	pH = 6.4	
CTRL	104 ± 6	
10 μM MK-801	82 ± 2	
10 μM MK-801, 10 μM CNQX	47 ± 3	*†‡
10 μM MK-801, 100 μM CNQX	25 ± 9	*†‡

require further study in intact systems and the testing of NMDA antagonist drugs in combination with drugs designed to block such other mechanisms.

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12. Oxygen-glucose deprivation was initiated by medium exchange to a deoxygenated solution containing (in mM) NaCl (116 for pH 7.4, 129.5 for pH 6.4), KCl (5.4), $MgSO_4$ (0.8), NaH_2PO_4 (1), $CaCl_2$ (1.8), Pipes (10), $NaHCO_3$ (15 for pH 7.4, 1.5 for pH 6.4), and phenol red (10 mg/liter). Exposure was terminated by medium exchange with oxygenated Eagle's minimum essential media (MEM, Earle's salts) at pH 7.4, supplemented with glutamine and glucose (final concentrations, 2 mM and 5.5 mM, respectively).
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22. The concentrations of glutamate antagonists used in the experiment summarized in Fig. 3 exceed those required to eliminate excitotoxicity in our system. The dissociation constant (K_d) for MK-801 binding to the NMDA receptor–gated channel is in the range of 37 nM (*14*); even on depolarized neurons, K_d is probably <500 nM [J. E. Huettner and B. P. Bean, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 1307 (1988)]. For 7-chlorokynurenate binding at the NMDA receptor glycine site K_d is 560 nM (*19*), and K_d for CNQX at the AMPA-kainate receptor is 0.3 to 1.5 μM (*15*).
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Common Forms of Synaptic Plasticity in the Hippocampus and Neocortex in Vitro

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Activity-dependent synaptic plasticity in the superficial layers of juvenile cat and adult rat visual neocortex was compared with that in adult rat hippocampal field CA1. Stimulation of neocortical layer IV reliably induced synaptic long-term potentiation (LTP) and long-term depression (LTD) in layer III with precisely the same types of stimulation protocols that were effective in CA1. Neocortical LTP and LTD were specific to the conditioned pathway and, as in the hippocampus, were dependent on activation of *N*-methyl-D-aspartate receptors. These results provide strong support for the view that common principles may govern experience-dependent synaptic plasticity in CA1 and throughout the superficial layers of the mammalian neocortex.

Activity-dependent synaptic plasticity in the mammalian brain is best understood in the CA1 region of the adult hippocampus, where conditioning stimulation of the Schaffer collateral pathway in vitro can induce *N*-methyl-D-aspartate (NMDA) receptor-dependent LTP (*1, 2*) and LTD (*3, 4*). An important question is whether what has been learned about the hippocampus can be applied generally to synaptic plasticity in the cerebral cortex. Although LTP has been demonstrated in the visual cortex (*5*), in the mature neocortex in vitro it has been reported to occur with low probability (*6–8*) and to require for induction pharmacological treatments to reduce inhibition (*9–11*) and stimulation patterns that vary substantially from those that are effective in the hippocampus (*6, 7, 12–14*). Similarly, the conditions for evoking LTD in the hippocampus may be very different from those in the neocortex. For example, in the hippocampus low-frequency stimulation evokes NMDA-dependent LTD (*3, 4*), whereas the same type of stimulation in the neocortex can yield LTP (*6*). Furthermore, strong stimulation in the presence of NMDA receptor antagonists causes LTD in the neocortex (*15, 16*) but no change in the CA1 region (*17*). In order to reconcile these disparate results, we directly compared the plasticity of synaptic responses evoked in adult rat hippocampal field CA1 with those evoked in adult rat and immature cat visual cortical layer III. To more closely approximate the stimulation-recording arrangement in the hippocampus (stimulating the Schaffer collaterals and recording in CA1), in neocortical preparations we stimulated the direct input to layer III from layer IV rather than using the traditional approach of stimulating the white matter (*18*).

In CA1, brief high-frequency (100 Hz) bursts of stimulation delivered to the Schaffer collaterals at the theta rhythm (5 to 7

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