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Ethanol, Stroke, Brain Damage, and Excitotoxicity

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CREWS, F. T., J. C. STECK, L. J. CHANDLER, C. J. YU AND A. DAY. *Ethanol, stroke, brain damage, and excitotoxicity*. PHARMACOL BIOCHEM BEHAV **59**(4) 981–991, 1998.—The N-methyl-d-aspartate (NMDA)-glutamate receptor could contribute to stroke, trauma, and alcohol-induced brain damage through activation of nitric oxide formation and excitotoxicity. In rat primary cortical cultures NMDA was more potent at activating nitric oxide formation than triggering excitotoxicity. Ethanol dose dependently inhibited both responses. In contrast, treatment of neuronal cultures with ethanol (100 mM) for 4 days signficantly increased NMDA stimulated nitric oxide formation and excitotoxicity. These findings suggest that ethanol acutely inhibits but chronically causes supersensitivity to NMDA-induced excitotoxicity in neuronal cultures. To investigate ethanol's interaction with stroke induced damage models of global cerebral ischemia were studied. Transient global ischemia resulted in a loss of hippocampal CA1 pyramidal neurons over a 3- to 5-day period. Determinations of the NMDA receptor ligand binding stoichiometry or postischemic receptor binding changes did not show differences between neurons that undergo delayed neuronal death following ischemia and those that show no toxicity, for example, CA1 and dentate gyrus, respectively. Acute ethanol (3 g/kg) was found to protect against ischemia-induced CA1 hippocampal damage by lowering body temperature, but not under temperature controled conditions. These studies indicate that the factors contributing to stroke-induced brain damage are complex, although they are consistent with chronic ethanol increasing stroke-induced brain damage by increasing NMDA excitotoxicity. © 1998 Elsevier Science Inc.

Ethanol Stroke Excitotoxicity NMDA Nitric oxide

HEAVY alcohol consumption can lead to cognitive and neurological impairments associated with decreases in cerebral volume (25). Alcohol consumption has also been implicated in increasing the risk for stroke-induced brain damage as well as in brain damage (59) due to traumatic brain injury (47). Recent studies have indicated that the major excitatory neurotransmitter, glutamate, may play a role in stroke, traumatic and alcohol-induced brain damage through a process referred to as excitotoxicity (17,19). Excitotoxicity occurs following excessive excitation of neurons through glutamate receptors that result in neuronal death.

Glutamate-mediated excitotoxicity has been divided into two components: a rapid component associated with osmotic swelling and often immediate neuronal death, and a delayed component that occurs as a progressive degenerative process over a period of hours to days. Both processes are related to the excessive accumulation of calcium (16,34,39,42). The slow progressive neuronal degeneration, i.e., delayed neuronal cell death, appears to involve the following events: glutamate depolarizes neurons leading to increased intracellular calcium ($[Ca^{2+}]_i$) through receptor-gated ion channels, particularly the glutamate *N*-methyl-D-aspartate (NMDA) receptors and voltage-sensitive calcium channels (14,16). Both glutamate and the increase in $[Ca^{2+}]_i$ are thought to trigger delayed neuronal death through the activation of phospholipases, proteases, and endonucleases (42). Thus, excitotoxicity is a central factor that contributes to the brain damage associated with stroke and traumatic brain injury.

Electrophysiological studies have shown that ethanol can inhibit NMDA–glutamate receptor-stimulated ion currents

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(29,30). These studies would suggest that ethanol might reduce excitotoxic effects if present during neuropathologic events (17,19). This is not consistent with the epidemiologic data on stroke that suggests increased risk for damage with both acute and chronic alcohol consumption 59). This article presents studies both in vitro and in vivo, which test complementary hypothesis regarding NMDA excitotoxiciy, stroke, and ethanol. Studies using cortical neuronal cultures test the hypothesis that acute ethanol will block NMDA-stimulated excitotoxicity and NMDA-stimulated nitric oxide formation, whereas chronic ethanol will sensitize neurons to these NMDA responses. Nitric oxide formation is implicated in neuronal toxicity because it forms highly oxidative metabolites that can be neurotoxic 21). Because NMDA excitotoxicity is implicated in stroke-induced brain damage, a second series of experiments test the hypothesis that acute ethanol can modify transient ischemia-induced brain damage. Although acute ethanol was found to block NMDA excitotoxicity and nitric oxide formation, in vivo studies on ischemic damage were found to be very complex and not inhibited by acute ethanol under well-controlled conditions. Chronic ethanol results in NMDA supersensitivity, which could contribute to increased brain damage following a stroke.

METHOD

Materials

All buffers, amino acids and salts were purchased from Sigma Chemical Co., St. Louis, MO. Platelet-derived horse syrum was obtained from GIBCO, Grand Island, NY. Ethanol was obtained from Aaper Alcohol and Chemical Co. Shelbyville, KY. *N*-methy-D-aspartate was from ICN Pharmaceuticals, Irvine, CA, and all radionucleotides were purchased from New England Nuclear, Boston MA.

Preparation of Neuronal Cultures

Cerebral cortical cultures were prepared from 1-day-old rat pups as previously described (7,9). Briefly, trypsin-dissociated cells were resuspended in Dulbrecco's modified Eagle's medium (DMEM) containing 25 mM glucose, 2 mM glutamine, and 10% plasma-derived horse serum (PDHS), and plated in 35 mm poly-L-Lysine precoated culture dishes at a density of 4×10^6 cells/dish. Cells were incubated at 37°C in a humidified incubator with 5% CO_2/95% air for 3 days and were then treated with 10 μM β-cytosine arabinoside (ARC) in fresh DMEM containing 10% PDHS for 2 days. Following this, the culture media was replaced with fresh DMEM containing 10% PDHS.

Measurements of Excitotoxicity

The procedure for examining NMDA-mediated excitotoxicity was based on a method previously described (9,12). After 96 h of incubation in the presence and absence of ethanol, the media was aspirated from the dishes and the cells were washed with 1 ml of Mg^{2+}-free HEPES–bicarbonate buffer (25 mM HEPES, 120 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 25 mM $NaHCO_3$, 10 μM glycine, 15 mM glucose, pH 7.4). Cultures were then incubated with or without 100 mM ethanol in 1 ml of HEPES–bicarbonate buffer for 10 min and then exposed to NMDA for 25 min. NMDA exposure was terminated by rapid aspiration of the buffer followed by washing the cells with 1 ml of DMEM. The cultures were then incubated with 1 ml of the glutamine/serum-free DMEM containing 5 mM glucose for 20 h. After the 20-h incubation period, neurotoxicity was measured by the amount of lactate dehydrogenase (LDH) released from the cells into the media using a spectrophotometric method (9,10). LDH release from cerebral cortical cultures has previously been shown to provide a linear relationship between the number of neurons destroyed and the amount of LDH activity in the media (10,13), and therefore provides a reliable estimate of neuronal damage that has been confirmed in cortical cultures using trypan blue and propidium iodide as markers of neuronal death (48).

Measurement of NOS Activity

NOS activity was determined by measuring the formation of [^{3}H]L-citrulline from cells preloaded with [^{3}H]L-arginine as described by Davda et al. (20). Cortical cultures were washed twice with 1 ml of HEPES buffer [140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 100 nm glycine, 15 mM glucose, and 25 mM HEPES (pH 7.4)] and incubated for 10 min in 1 ml of HEPES buffer. [^{3}H]L-Arginine (3 Ci/ml) was then added to each culture dish and allowed to incubate for 5 min followed by addition of agonist. After 5 min of stimulation, incubations were terminated by the rapid aspiration of the incubation buffer, two washes with 1 ml of ice-cold stop buffer (118 mM NaCl, 4.7 mM KCl, 1.18 mM KH_2PO_4, 24.8 mM $NaHCO_3$, 4 mM EDTA, 5 mM L-arginine), followed by the addition of 1 ml of 0.3 M $HClO_4$. After 10 min, the cell extract was neutralized with 3.0 M K_2CO_3 and 50 μl aliquots of the tissue extract taken for determination of total uptake of [^{3}H]L-arginine by liquid scintillation spectroscopy. To determine formation of [^{3}H] citrulline from [^{3}H]L-arginine, a 500 μl aliquot of the neutralized extract was applied to columns containing 2 ml of a slurry of Dowex AG50WX-8 (Na^+ form) and H_2O (1:1), washed three times with 2 ml of H_2O and the eluent (6 ml) collected into scintillation vials. [^{3}H]Citrulline formation was expressed as a percent conversion from the total [^{3}H]arginine taken up into the cells.

Ethanol Treatment

Neuronal primary cultures were treated with 100 mM ethanol and placed in a Nalgene zip lock bag along with a beaker containing 500 ml of 100 mM ethanol to maintain the ethanol concentration in the dishes at 100 mM. The bags were filled with 5% CO_2/95% air and returned to the incubator for an additional 96 h. The ethanol concentration in the culture media after treatment was 83.7 ± 2.7 mM. Control dishes were treated identically but were not exposed to ethanol.

Autoradiographic Binding Methods

Coronal sections of frozen brains, 6 mm in thickness, were cut on a cryostat (Hacker-Bright) at −20°C, thaw mounted onto gelatin-coated slides, and used for autoradiography or histological stains. [^{3}H]-cis-4-phoshonomethyl 2-piperidine carboxylic acid ([^{3}H]CGS) was assayed as described by (48); 5-methyl-10,11-dihydro-5H dibenzo [a,d] cyclohepten-5,10 imine maleate ([^{3}H]MK-801) binding as described previously (48); [^{3}H]GLU was assayed using a modified version of the method of Westerberg (57). [^{3}H]AMPA receptors binding sites were quantified using Murphy's et al. method (36). [^{3}H]Kainate receptor binding sites were quantified using as reported previously by Westerberg et al. (57). [^{3}H]PK-11195 binding was performed as described previously (54).

Ischemia

Male Wistar rats (250–300 g) were subjected to 20 min of global ischemia through four vessel occlusion as described by

Pulsinelli (44). Briefly, the neck was opened and the common carotid artery's were occluded with small clips for 20 min. Body temperature was maintained at 37°C during the surgery and the postoperative recovery period with a rectal thermometer and a heating lamp. Bilateral carotid occlusion for 5 min in Mongolian gerbils was performed as described previously (28). Sham animals were handled identically to the ischemic animals with the exception of carotid occlusion.

All animal procedures were approved by the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee and are in accordance with the NIH "Guide for the Care and Use of Laboratory Animals."

Data and Statistical Analysis

Binding data were analyzed by computer calculation of the line of best fit using the Rosenthal conversion for K_d and B_{max} values (35,50). The resulting data were analyzed by Hill plots by plotting $\log_{10} B/B_{max} - B$ vs. log of the concentration of radioligand.

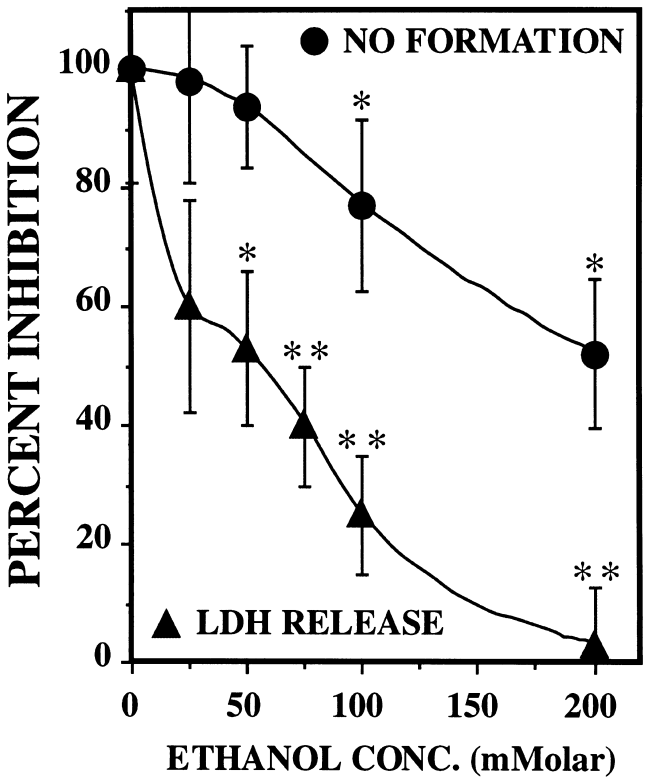

FIG. 1. Effects of ethanol on NMDA-stimulated excitotoxicity (lactate dehydrogenase release—LDH) and nitric oxide (NO) formation. For LDH release neuronal cultures were preincubated in Mg^{2+}-free HEPES buffer with the indicated concentration of ethanol 10 min prior to addition of 100 μM NMDA for 25 min. LDH activity was measured in the media 20 h following NMDA and ethanol wash out. NO formation was determined by measuring the percent [^{3}H]citrulline formed from [^{3}H]arginine. Neuronal cultures were incubated in the presence of 1 mM Mg^{2+} in the presence of various concentrations of ethanol. NMDA (25 μM) treatment was for 5 min. Values represent the means ± SEM of at least four experiments each determined in triplicate. Asterisks indicates significant difference from control values $^*p < 0.05$; $^{**}p < 0.01$ [see Chandler et al. (7,9)**<Q6>** for more details].

Values were subjected to two-way ANOVA. Post hoc comparisons for all interactions or main effects with $p < 0.05$ were made using Newman–Keuls analysis and judged to be significantly different.

RESULTS

In Vitro Studies

Delayed neuronal death and excitotoxicity can be investigated using primary neuronal cultures. *N*-methyl-D-aspartate (NMDA) exposure for as little as 5–30 min triggers delayed neuronal death that is not apparent until hours latter and reaches a maximum between 16 and 20 h later 10,48). The NMDA dose–response curve is extremely sharp, for example, from no effect at 10 μM to a maximal response at 100 μM (EC_{50} = 54 μM). Ethanol added during NMDA treatment results in a concentration-dependent inhibition of NMDA-mediated toxicity with approximately 38% inhibition produced by 25 mM ethanol and essentially complete inhibition at 200 mM ethanol (IC_{50} = 60 mM) (Fig. 1) (10). This reduction by acute ethanol was also observed under phase-contrast microscopy as a lack of development of granulation and a sparing of disintegration of neuritic processes (19). These studies indicate that NMDA receptor activation triggers a delayed neuronal death that progresses over several hours and that ethanol can inhibit NMDA excitotoxicity in vitro. This could not explain the epidemiological data indicating that ethanol appears to be associated with an increased incidence of brain damage in stroke and trauma injury.

Mechanistic studies into the actions of ethanol on NMDA-mediated excitotoxicity were extended to studies on NMDA-stimulated nitric oxide (NO) formation. NO is a gas that is formed by nitric oxide synthetase, an enzyme that converts arginine to NO and citrulline. NO has been implicated in NMDA excitotoxicity in primary neuronal cultures (21) and in vivo models of stroke-induced brain damage (38). NO is known to be highly oxidative and acute oxidation might tigger processes that lead to progressive delayed neuronal death (19). These studies prompted experiments on the effects of ethanol on NMDA-stimulated NO formation in cerebral cortical cultures (7). Glutamate stimulated NO formation, but

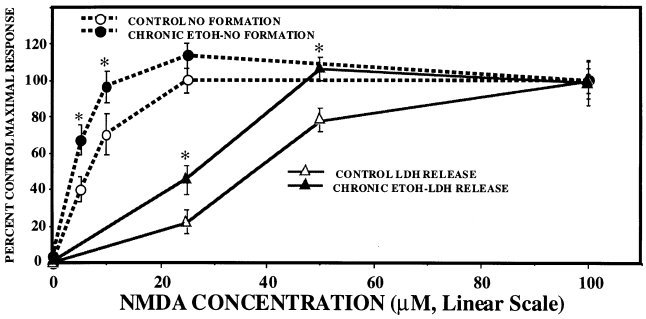

FIG. 2. Effects of chronic ethanol treatment on NMDA-stimulated excitotoxicity and nitric oxide formation. Both responses were tested in cerebral cortical neuronal cultures, for example, control and chronic ethanol (100 mM^{-4} days) and were tested under the same conditions. Note that the NO response is significantly more sensitive to NMDA (EC_{50} ~7 μM, with a maximal response at ~25 μM for NO, compared to excitotoxicity—EC_{50} ~40–50 μM with a maximal response at ~100 μM NMDA). Both responses show a left shift in the dose–response curve following chronic ethanol treatment.

not cholinergic, adrenergic, purinergic, and peptidergic agonists (7). Characterization of the glutamate response with selective agonists reveals that NMDA, kainate, and AMPA all stimulate NOS activity with a relative maximal efficacy of NMDA >> kainate > AMPA. Acute ethanol treatment in the presence of physiological concentrations of Mg^{2+} inhibits NMDA-stimulated NO formation at fairly high concentrations of ethanol (Fig. 1). The significantly greater potency of ethanol at inhibition of excitotoxicity than at inhibition of NO formation may be related to different subsets of NMDA receptors being involved in each of these responses. In any case, glutamate NMDA receptors appear to be the predominant receptor coupled to NO formation as well as excitotoxicity in primary neuronal cultures and perhaps throughout the brain (7). Although these studies suggest that ethanol might have a protective effect, ethanol treatment of neuronal cultures was only for a few minutes. It is difficult to determine the duration of ethanol consumption in epidemiological studies linking ethanol to increased brain damage.

Studies have found that heavy alcohol consumption over a period of years can lead to cognitive and neurological impairments as well as enlargement of the ventricles, increases in

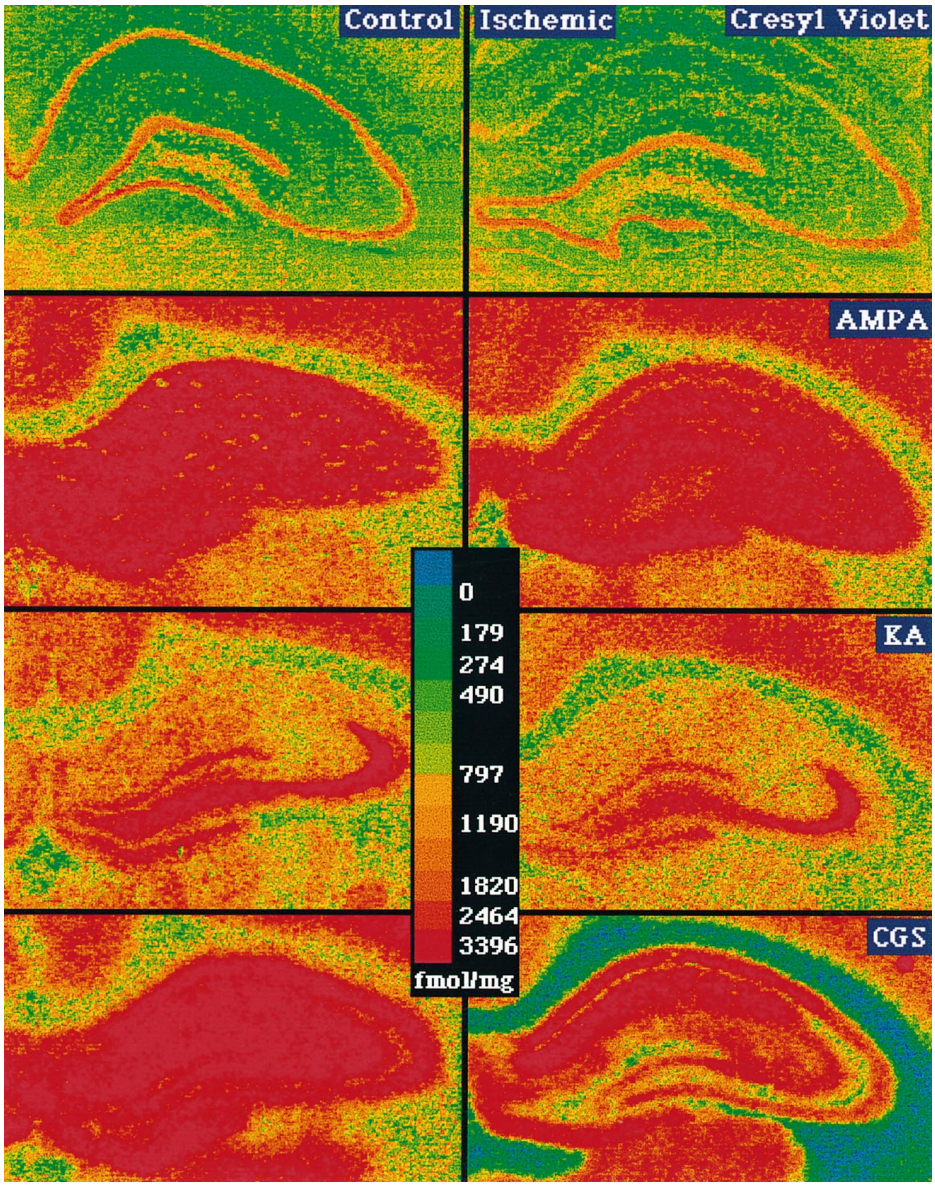

FIG. 3. Effect of ischemia on [^{3}H]AMPA, [^{3}H]kainate, and NMDA [^{3}H]CGS binding in rat hippocampus. Top panels are imaged and colorized cresyl violet-stained sections showing hippocampal pyramidal neuron regions 7 days after surgery in sham-operated controls (left) and ischemic animals (right). Note the loss of CA1 neurons. The change in shape of the dentate gyrus is due to sections at slightly different sites. Transient global ischemia was induced for 20 min through four-vessel occlusion as described by Pulsinelli (44) and animals allowed to recover for 7 days. Binding analysis was performed with saturating concentrations of ligands as described in the Method section. All frames are related to color scale quantitation.

cortical spaces, and decreased brain cellular mass (25). It has been suggested that neurodegeneration in chronic alcoholics is related to excitotoxicity (17,19), and this might also be involved in stroke and traumatic brain damage. Neuronal primary cultures exposed to ethanol (100 mM) for 4 days have no morphological or clear biochemical (as determined by total protein and LDH activity per well) effects on primary neuronal cultures. However, withdrawal of ethanol followed by a short NMDA exposure, significantly increases NMDA stimulated delayed neuronal death (Fig. 2) (9). Excitotoxic neuronal death was increased by 36 and 22% in response to 25 and 50 μM NMDA, respectively. Fine dendritic processes appear especially sensitive to NMDA-induced damage, which is not surprising because NMDA receptors tend to localize on neuronal dendrites. In contrast to the effects of withdrawal after chronic ethanol on NMDA neurotoxicity, inclusion of ethanol (100 mM) during the NMDA exposure period significantly reduced LDH release by approximately 50% in both control and chronically treated cultures. These results indicate that chronic exposure of ethanol to cerebral cortical neurons in culture can sensitize neurons to excitotoxicity during ethanol withdrawal and also highlight the fact that the apparent threshold for triggering delayed neuronal death is decreased because concentrations of NMDA, for example, 25 μM, that previously did not trigger significant excitotoxicity do trigger neuronal death in ~36% of neurons following chronic ethanol treatment. These studies strongly support the hypothesis that chronic ethanol enhances excitotoxicity as well as suggesting that the threshold required to trigger the process of delayed neuronal death is decreased.

To investigate the mechanism of enhanced excitotoxicity the effects of chronic ethanol treatment on NMDA stimulated NO formation were studied. Chronic (4 days) treatment of primary cerebral cortical cultures with ethanol (100 mM) potentiated NMDA-stimulated and KCl (30 mM)-stimulated NO formation (8). In contrast, chronic ethanol had no effect upon either kainate, AMPA, or ionomycin-stimulated NO formation. NMDA was potentiated at submaximal concentrations (5 and 10 μM) with no increase at maximal concentrations (25 μM and higher). Thus, acute ethanol inhibits receptor responses and chronic ethanol potentiates NMDA receptor responses.

The potentiation of NMDA-induced NO formation and excitotoxicity likely represent an increase in NMDA receptor sensitivity. Chronic ethanol does not alter NOS protein levels or NOS sensitivity to calcium or neuronal sensitivity to other glutamate agonist activated NO formation (11). Other studies have found that chronic ethanol increases NMDA-stimulated calcium flux in neuronal cultures (24,26) consistent with NMDA receptor supersensitivity and increased NO and excitotoxicity responses. The mechanism of NMDA supersensitivity is not clear. The NMDA receptor is composed of multiple subunits including an obligatory NR1 subunit that has multiple splice varients and NR2A-D subunits that confer functional heterogeneity to NMDA receptors (18). Studies examining the levels of NMDA subunits following chronic ethanol treatment have yielded conflicting results, with some studies showing changes (24) and others no change (11,18). Studies in cortical cultures done essentially identical to those reported here found no change in NMDA receptor binding or protein levels of NR1 or the modulating NR2A or NR2B subunits, the predominant subunits in cortical cultures (11). Both tyrosine and serine/threonine kinases as well as phosphatases can modulate NMDA receptor function in a manner consistent with supersensitivity (18). Although the mechanism of NMDA receptor supersensitivity is not clear, the acute inhibition of NMDA excitotoxicity and NO formation, with a resulting supersensitivity with chronic treatment, likely represent important actions of ethanol that could alter neurotoxic insults in vivo.

In Vivo Studies

A significant portion of brain damage that occurs following stroke-induced cerebral ischemia is due to glutamate triggered excitotoxic delayed neuronal death (60). Because our in vitro studies suggest that ethanol modifies these processes, in vivo models of stroke in rats and gerbils were used to investigate the interaction of ethanol and stroke-induced brain damage. Transient global ischemia was produced in rats for 20 min by four vessel occlusion (45). Histopathology was monitored by cresyl violet and hematoxyline-eosin stains. At the end of ischemia no histologic damage was found. At 3 and 7 days postischemia CA1–hippocampal neurons consistently showed almost complete neuronal loss with minor damage to CA3 and CA4 and no detectable damage to dentate gyrus (Figs. 3 and 5). This in vivo delayed neuronal death that occurs after transient ischemia is comparable to that found in neuronal culture following short-term treatment with NMDA. The lack of pathology in hippocampal–dentate gyrus compared to the almost complete loss of neurons in hippocampal–CA1 is similar to that found in other studies and prompted experiments into the nature of the differences in neuronal sensitivity to ischemia. It is possible that the sensitivity of the CA1 region to ischemia-induced delayed neuronal death is due to a greater density or unique subtype of heteroligomeric NMDA receptor containing unique subunits. B_{max} and K_d values for [^{3}H]MK-801 a noncompetitive antagonist, [^{3}H]GLU an

TABLE 1

COMPARISON OF HIPPOCAMPAL CA1 AND DENTATE GYRUS BINDING CONSTANTS

	[^{3}H]CGS		[^{3}H]GLU		[^{3}H]MK-801	
Hippocampal	B_{max} (pmol/mgP)	K_d (NM)	B_{max} (pmol/mgP)	K_d (NM)	B_{max} (pmol/mgP)	K_d (NM)
CA1 stratum oriens	6.7 ± 0.5	88 ± 8	3.9 ± 0.1	86 ± 8	1.7 ± 0.09	42 ± 2
CA1 stratum radium	9.4 ± 0.9	117 ± 6	5.0 ± 0.2	98 ± 11	1.9 ± 0.06	44 ± 1
Dentate gyrus						
Int.	8.2 ± 0.9	117 ± 10	4.7 ± 0.07	103 ± 9	1.6 ± 0.06	44 ± 2
Ext.	7.8 ± 0.9	123 ± 15	4.8 ± 0.1	108 ± 9	1.6 ± 0.05	41 ± 2

Values shown are B_{max} and K_d determinations from regression analysis of saturation isotherms done on six separate animals. Auto radiographic binding was determined as described in the Method section.

NMDA agonist, and [^{3}H]CGS, a competitive NMDA antagonist, were all found to be similar in CA1 and dentate gyrus (Table 1). Thus, the density of receptors appears to be the same. As mentioned above, multiple subunits, for example, NR1 and NR2A-D, may form subtypes of receptors. Immunoprecipitation studies with subunit specific antibodies have indicated that the NMDA–receptor ion channel in cerebral cortex is heteromeric containing both NR2A and NR2B as well as NR1 subunits (51). Expression studies have indicated that NR1 or NR2 expression alone does not yield competitive antagonist, for example, CGS or noncompetitive antagonist, for example, MK-801, binding sites (31,32). Combined expression of NR1 and NR2 subunits yields receptors possessing GLU-agonist, CGS-competitive antagonist and MK-801 sites with different affinities (28,31,32). NR1–NR2A constructs have prominent high affinity CGS-competitive antagonist sites (28,31), whereas NR1–2B constructs show greater polyamine sensitivity (32). The [^{3}H]CGS/[^{3}H]GLU/[^{3}H]MK-801 B_{max} ratio of 5/4–3/1 found in both CA1 regions and dentate gyrus of hippocampus is consistent with an NR1, NR2A–NR2B com-

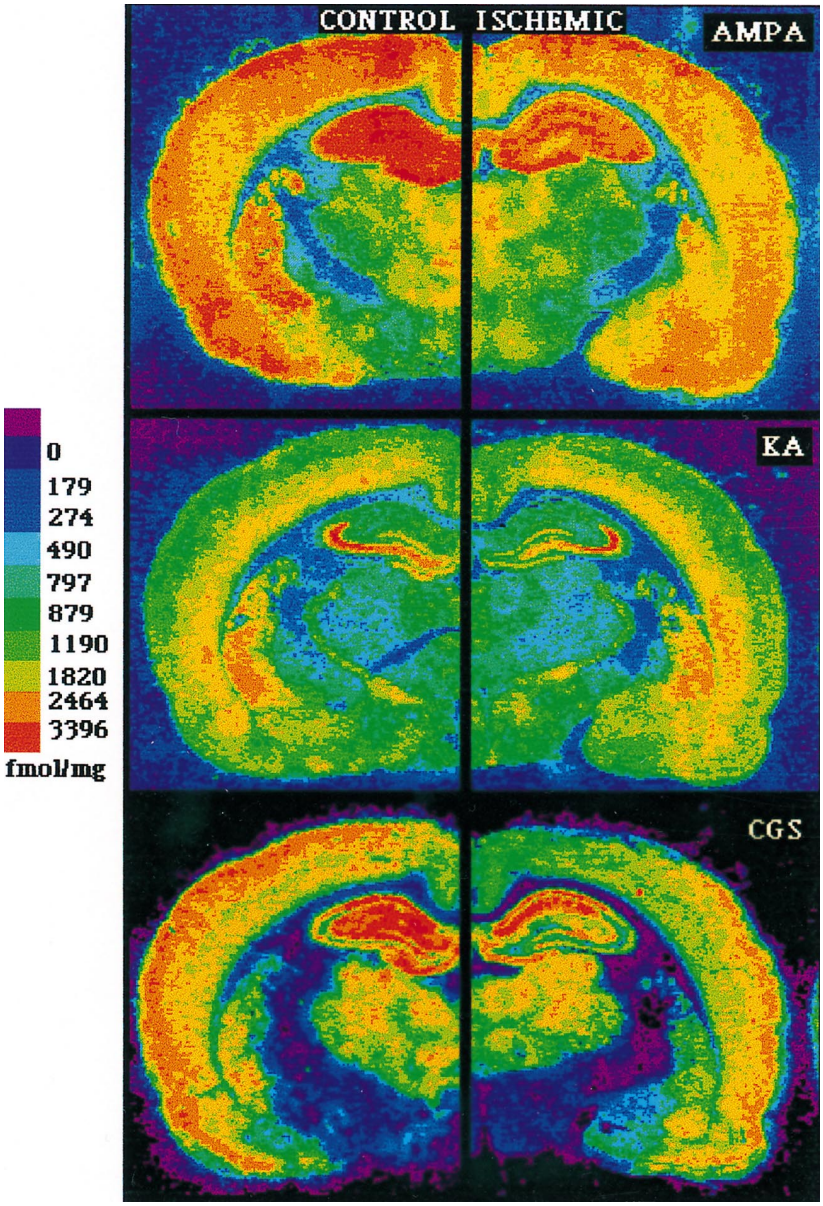

FIG. 4. Effect of ischemia on glutamate receptors [^{3}H]AMPA, [^{3}H]kainate, and NMDA[^{3}H]CGS binding in rat brain. Autoradiographic binding of receptor ligands was performed 7 days after transient global ischemia (right) and is compared to sham-operated controls (left). Transient global ischemia was induced for 20 min through four-vessel occlusion as described in the Method section, and animals allowed to recover for 7 days. Binding analysis was performed with saturating concentrations of ligands. All frames are related to color scale quantitation.

plex that is similar to that found in neuronal cortical cultures. Taken together, these data suggest that the unique sensitivity of CA1 to delay neuronal death following ischemia is not due to a unique subtype of NMDA receptor.

NMDA receptor binding was also investigated at several time points after ischemia to determine if specific sites were lost with the loss of neurons. At 7 days postischemia [^{3}H]CGS (Figs. 3 and 4) and [^{3}H]MK-801 binding decreased ~30–40%, whereas [^{3}H]GLU decreased ~20%. [^{3}H]AMPA and [^{3}H]Kainate decreased ~40% in most brain regions studied (Figs. 3 and 4). These changes were remarkably similar across all hippocampal regions and cerebral cortex. This was particularly surprising, considering that there was significant neuronal loss in CA1 but not cell loss in hippocampal dentate gyrus or many other areas showing large decreases in receptor density (Figs. 3 and 4). The significant neuronal loss of greater than 90% of the CA1 pyramidal cells with much smaller changes in NMDA receptor binding sites suggests that many of the NMDA receptor binding sites in hippocampal CA1 are on interneurons and/or presynaptic sites. These studies suggest that ischemia reduces

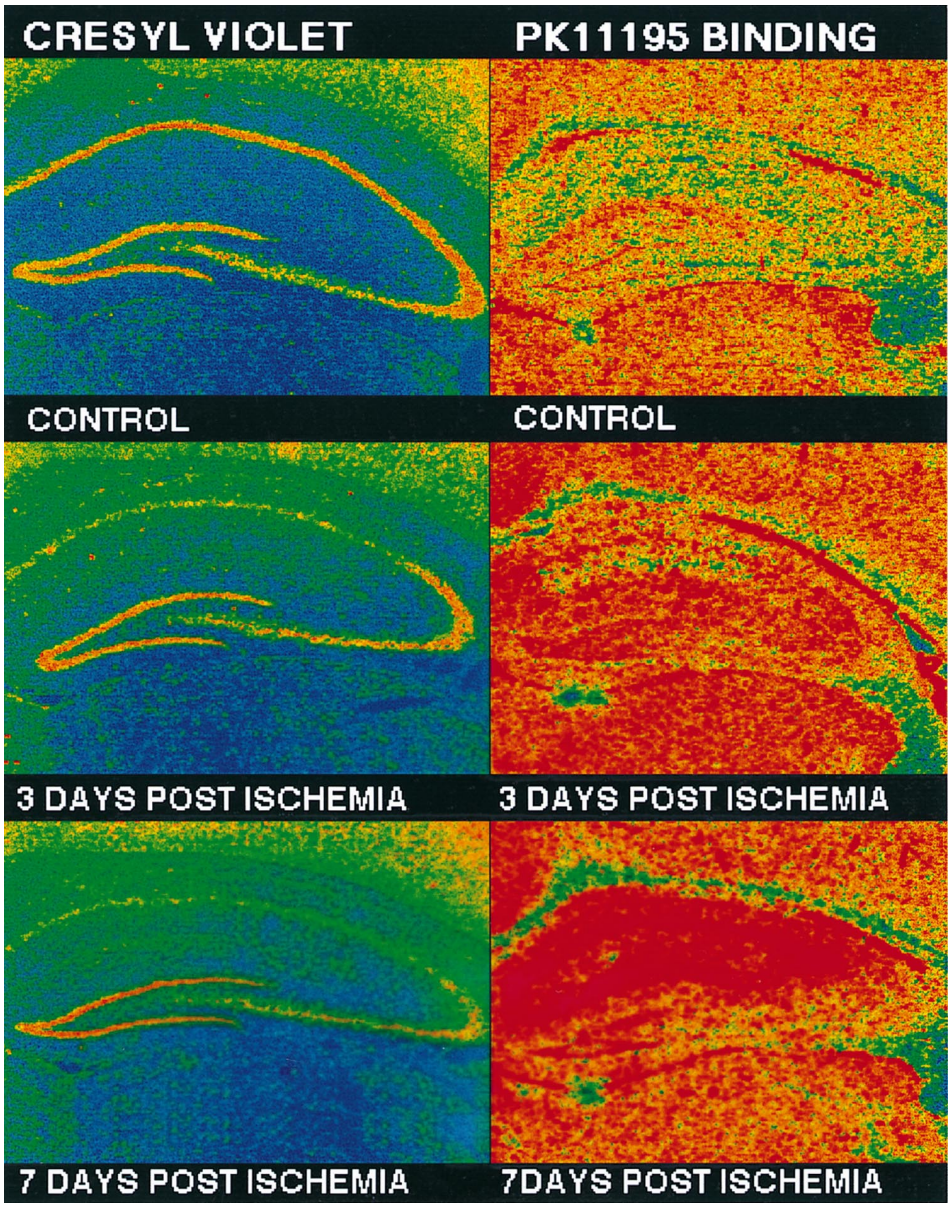

FIG. 5. Comparison of histology and [^{3}H]PK11195 binding in the gerbil hippocampus following ischemia. Shown are hippocampal sections stained with cresyl violet, imaged, and colorized to show pyramidal layers (left) and [^{3}H]PK11195 binding densities from autoradiographs (right). Gerbils were exposed to 5 min of bilateral carotid occlusion as described in the Method section. Shown are control sham operated, and 3 and 7 days postischemic times. Sham controls were identical to 0 time postischemia animals. Body temperature during ischemia was monitored with a rectal thermometer and maintained at 37°C with a warm pad and heat lamp.

TABLE 2

EFFECTS OF ETHANOL AND BODY TEMPERATURE ON HIPPOCAMPAL CA1 NEURON DENSITY AND [^{3}H]-PK11197 BINDING

	Controls Sham 37.0 ± 0.4°C		Controls Ischemia 37.2 ± 0.7°C		Ethanol (3 g/kg) Ischemia 33.9 ± 0.5°C		Ethanol (3 g/kg) Ischemia 37.2 ± 0.8°C	
Body Temp.	Neuron Cts.	[^{3}H]PK11195 Binding	Neuron Cts.	[3H]PK11195 Binding	Neuron Cts.	[3H]PK11195 Binding	Neuron Cts.	[3H]PK11195 Binding
Day 0	98 ± 2	105 ± 5	99 ± 2	99 ± 3	88 ± 9	101 ± 12	101 ± 7	93 ± 3
Day 3	102 ± 9	88 ± 9	42 ± 12*	157 ± 6*	107 ± 8	94 ± 8	21 ± 14*	168 ± 34*
Day 7	104 ± 4	101 ± 12	3 ± 2*	359 ± 65*	94 ± 12	105 ± 9	20 ± 22*	313 ± 38*

Shown are the values for hippocampal CA1 neuronal counts (neuronal nuclei in cresyl violet-stained sections/250 μM) and [3H]-PK11195 binding (dpm) to stratum radiatum of CA1 hippocampal sections. Gerbils were exposed to 5-min ischemia by bilateral carotid occlusion. Sham controls were subject to the same procedures without occlusion of the carotids. Body temperature was monitored by a rectal thermometer and maintained by a heating pad and heat lamp when necessary. Values represent the mean ± SEM of 5–12 determinations.

*$p < 0.01$ compared to sham control.

glutamate receptors throughout the brain at 7 days postischemia. No relationship was found between receptor density or changes in density that could be related to the almost complete cell loss in CA1 and lack of histopathology in dentate gyrus. These findings suggest that the selective loss of hippocampal CA1 is not due to a unique set or high density of glutamate receptors.

The fact that acute ethanol inhibited and chronic ethanol sensitized to excitotoxicity in neuronal cultures prompted experiments on ischemic brain damage in vivo. The rat model of global ischemia was problematic due to high mortality when ethanol was administered, whereas the gerbil model of global ischemia, which is more commonly used, did not suffer from high mortality when animals were treated with ethanol. Two induces of brain damage were studied; histological examination of neuron counts, and binding of [^{3}H]PK11195 an index of microglial reactivity and brain damage. [^{3}H]PK11195 binding has been localized to reactive microglia that increase within damaged areas of brain following ischemic insults as well as other neuropathological conditions. [^{3}H]PK11195 binding coorelates with neuronal damage and provides a quantitative measure of neuronal damage (37,54). Neurons in hippocampal CA1 decreased from sham values of 98 ± 13 neurons/250 μm to 3 ± 2 neurons/250 μm at 7 days postischemia (Fig. 5 and Table 1). [^{3}H]PK11195 binding increased to more that 300% of control values in CA1 at 7 days postischemia (Fig. 5 and Table 2) and also increased in other brain areas where histological neuronal counts are more difficult to quantitative (Fig. 6). Thus, in both rats and gerbils short periods of ischemia result in pronounced damage to hippocampal CA1, but not dentate gyrus.

To determine if ethanol could alter stroke-induced brain damage, gerbils were treated with 3 g/kg ethanol IP 15 min prior to ischemia. Treatment with ethanol was found to protect against both neuronal loss of CA1 and the increase in [^{3}H]PK11195 binding associated with brain damage. However, hypothermia has been shown to protect against ischemia and animals treated with ethanol prior to ischemia had a reduction in body temperature of approximately 3°C. These finding prompted experiments where body temperature was maintained similar to that of controls. Maintaining body temperature in alcohol-treated animals resulted in a loss of the protective effect of ethanol with neuronal loss and increased [^{3}H]PK11195 binding essentially identical in ischemic controls with a normal body temperature and ethanol-treated animals where body temperature was maintained (Table 2). These findings suggest that the protective effect of ethanol is related to hypothermia and not other mechanisms.

DISCUSSION

The ability of ethanol to block NMDA-stimulated excitotoxicity and NO formation in primary cortical cultures is consistent with studies that indicate that ethanol can block NMDA receptors. Whole-cell patch-clamp studies in dissociated hippocampal neurons in culture found that low concentrations of ethanol can inhibit NMDA-induced ion currents (29). These studies have been extended to neurons in slices from adult animals with similar inhibition (30), and to in vivo recordings where most NMDA responses have been found to be sensitive to ethanol (53). NMDA-stimulated NO formation is likely due to the direct activation of NOS by calcium. In cerebellar granule cells in culture, ethanol has been found to inhibit NMDA calcium flux (26). Excitotoxicity is thought to be triggered by processes activated by excessive calcium flux (17,19). Thus, it is reasonable to suggest that ethanol, by reducing NMDA-stimulated calcium influx, blocks NMDA excitotoxicity.

Chronic ethanol enhanced NMDA-stimulated NO formation and excitotoxicity, particularly at low concentrations of NMDA. Excitotoxic neuronal death involves a cascade of events occurring over several hours following a few minutes of exposure to NMDA activation. Although a number of sites of increased sensitization could be involved, NMDA-NO supersensitivity is a rapid response. The similarity of NMDA–NO supersensitivity to NMDA excitotoxicity supersensitivity suggests that excitotoxic supersensitivity, at least in part, occurs during the initial NMDA receptor activation. This is consistent with findings in cerebellar granule cells that found chronic ethanol increased NMDA induced increases in intracellular calcium as determined using fura-2 (26). These studies suggest that chronic ethanol induces an adaptive supersensitivity of NMDA receptors that can increase excitotoxicity, particularly at low levels of stimulation. The association of ethanol with stroke-induced brain damage could be related to ethanol induced increases in excitotoxicity.

Excitotoxicity likely makes a major contribution to stroke and trauma-induced brain damage. Hippocampal CA1 neurons are particularly sensitive to ischemic damage. Glutamate is likely involved in ischemic hippocampal CA1 damage because lesions of the perforant path (58) blocking excitatory in-

puts to the hippocampus or lesions of the CA3 to CA1 glutamate projections (41) protect against global ischemia-induced CA1 neuronal death. Our findings of high NMDA receptor density throughout the hippocampus suggest that other factors are involved in the greater sensitivity of hippocampal CA1 to ischemic damage compared to hippocampal dentate gyrus. AMPA and kainate receptors were measured and could be involved in the glutamate response. AMPA receptors were similar in dentate gyrus and CA1, whereas kainate receptors were not present in CA1. How a lack of kainate receptors might sensitize to ischemic damage is not clear.

Studies of postischemic changes in receptors indicated that glutamate receptor density decreased throughout the brain and did not follow any specific histopathological neuron loss observed in CA1 or any other brain region. Previous studies have reported decreased binding of [^{3}H]TCP, which likely binds to the same sites as [^{3}H]MK-801, and [^{3}H]CPP following global ischemia similar to our findings (40). In another study using the same model of global ischemia we did reported a 60% decrease in AMPA binding and 25% decrease in [^{3}H]GLU sites in CA1 following global ischemia (57). Similar to our findings, decreased receptor binding was widespread

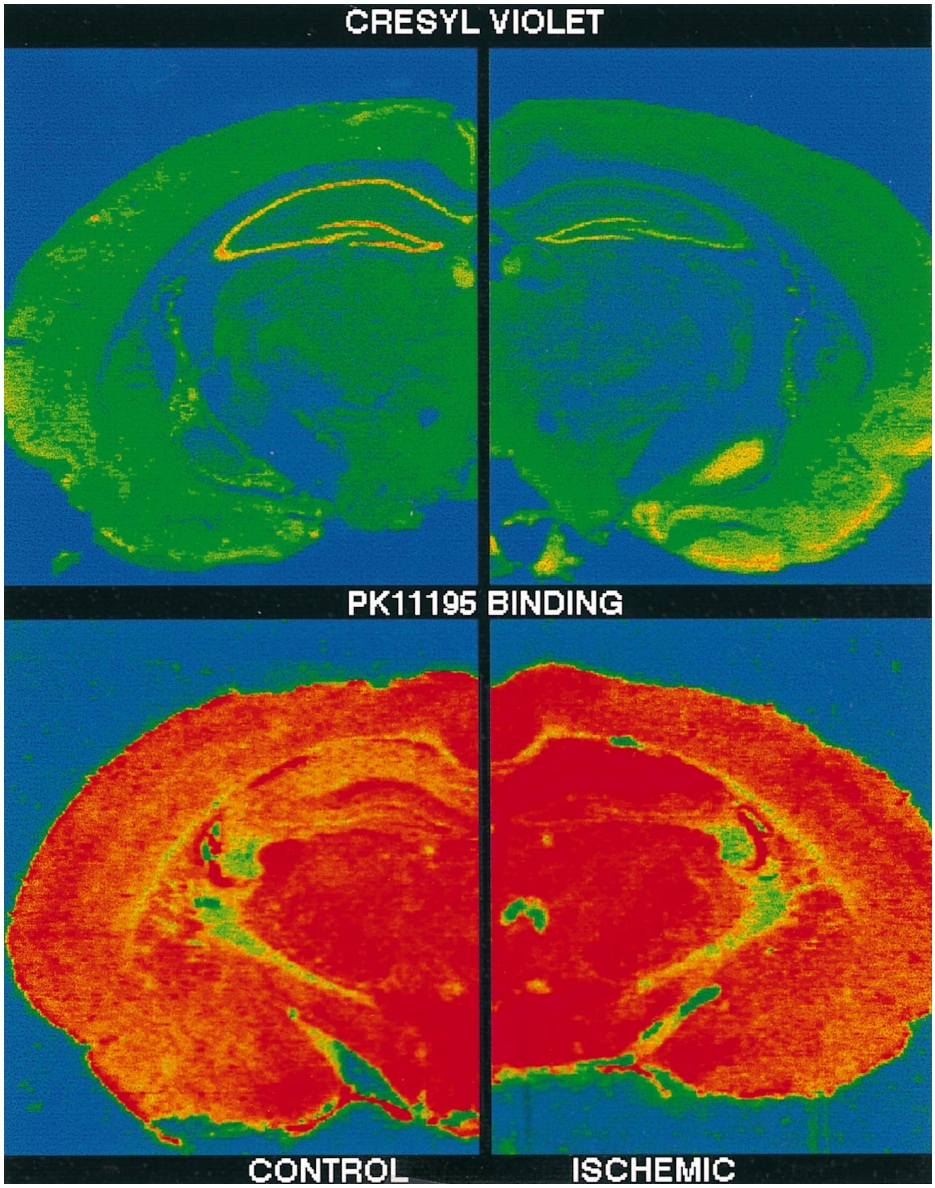

FIG. 6. Comparison of histology and [^{3}H]PK11195 binding in the gerbil brain following ischemia. Shown are coronal sections stained with cresyl violet (top) and [^{3}H]PK11195 binding densities from autoradiographs (bottom). Gerbils were exposed to 5 min of ischemia by bilateral carotid occlusion as described in the Method section. Data shown are representative for controls (left) and 7 days postischemic time (right). Sham controls were the same as 0 time postischemia values. Body temperature was monitored with a rectal thermometer and maintained at 37°C by a heating pad and heating lamp for both groups.

and did not correlate with histopathology. [^{3}H]AMPA and [^{3}H]GLU returned to control levels 4 weeks postischemia in dentate gyrus, consistent with ischemia inhibiting receptor protein synthesis throughout the brain, resulting in a transient decline in glutamate receptors across cortical and hippocampal regions independent of histopathology (57). These studies suggest that changes in glutamate receptor density are triggered in most neurons following ischemia, whereas delayed neuronal death occurs in only certain susceptible neurons. Chronic ethanol by increasing sensitivity to glutamate likely increases sensitivity to both the decreases in receptors as well as the loss of neurons.

Transient global ischemia, which occurs clinically in patients surviving cardiac arrest, causes irreversible injury to a few specific populations of neurons, particularly hippocampal CA1. Delayed neuronal death of hippocampal CA1 following global ischemia has been reported in the rat (45) the gerbil (27), and in humans (43). NMDA receptor blockage by competitive antagonists (52,55) or noncompetitive antagonists such has MK-801 have been reported to protect against CA1 damage in both the rat (15,23,46,56) and gerbil (3,22,33,46) in most but not all studies (4). Ethanol blocks NMDA receptor responses including excitotoxicity, suggesting that it might protect against transient ischemia. Ethanol (3 g/kg) lowered body temperature during ischemia and did show protection against global ischemia-induced delayed CA1 neuronal death. Ethanol is well known to lower body temperature dependent upon the ambient temperature. A 2–4°C decline in brain temperature during or following transient ischemia in rats has been reported to significantly attenuate ischemic brain damage (5,6). This prompted a series of experiments where body temperature was carefully controlled. When body temperature was maintained, no significant protection was found. Thus, ethanol (3 g/kg) induced hypothermia will protect against cerebral ischemia.

There are several possible explanations for the lack of ethanol protection when body temperature is maintained at 37°C. Although it is possible that the dose of 3 g/kg was not sufficient to block NMDA receptors, previous studies have indicated that as little as 0.75 g/kg ethanol can block NMDA responses in vivo (53). However, the possibility that NMDA blockade was not sufficient can not be ruled out. Another possible explanation involves ethanol induced changes in intracellular magnesium ($[Mg^{2+}]_i$). Ethanol has been shown to rapidly decrease $[Mg^{2+}]_i$ in both in vitro (2) and in vivo experiments (1). Mg^{2+} inhibits NMDA receptors through a voltage-dependent block. Further, Mg^{2+} enhances ethanol inhibition of NMDA-stimulated NO formation. These findings raise the possibility that ethanol-induced decreases in $[Mg^{2+}]_i$ may blunt ethanol inhibition of NMDA receptors. Although in vivo electrophysiological studies have shown ethanol inhibition of NMDA responses, not all neurons show inhibition (53). It is possible that under ischemic conditions decreases in $[Mg^{2+}]_i$ blunt the inhibitory actions of ethanol on NMDA receptors. Another complicating factor involves studies finding no protective effects of MK801 and other NMDA antagonists on global ischemia-induced loss of hippocampal CA1 neurons. A recent study has suggested that MK801-induced hypothermia is responsible for the protective effect of NMDA antagonists and not direct receptor inhibition of CA1 NMDA receptors (4). Regardless of the mechanism, the lack of protective effect of ethanol on global ischemia-induced brain damage is consistent with epidemiological studies, suggesting that ethanol is a risk factor in stroke and trauma-induced brain damage. NMDA supersensitivity following 4 days of ethanol treatment of cortical cultures would be expected to contribute to increased ischemic brain damage if this occurs in vivo. Although some studies have found chronic ethanol-induced increases in NMDA receptor binding in specific brain regions (18), this is not the case for all studies (49). However, chronic ethanol-induced supersensitivity can occur in the absence of a change in receptor binding or NMDA subunit composition (11). Thus, the epidemiological association of increased stroke pathology with acute and chronic alcohol abuse may be due to a lack of acute protective action as well as a sensitization during prolonged abuse.

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