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Comparison of Cannabidiol, Antioxidants, and Diuretics in Reversing Binge Ethanol-Induced Neurotoxicity

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Abstract

Binge alcohol consumption in the rat induces substantial neurodegeneration in the hippocampus and entorhinal cortex. Oxidative stress and cytotoxic edema have both been shown to be involved in such neurotoxicity, whereas *N*-methyl-D-aspartate (NMDA) receptor activity has been implicated in alcohol withdrawal and excitoxic injury. Because the nonpsychoactive cannabinoid cannabidiol (CBD) was previously shown in vitro to prevent glutamate toxicity through its ability to reduce oxidative stress, we evaluated CBD as a neuroprotectant in a rat binge ethanol model. When administered concurrently with binge ethanol exposure, CBD protected against hippocampal and entorhinal cortical neurodegeneration in a dose-dependent manner. Similarly, the common antioxidants butylated hydroxytoluene and *a*-tocopherol also afforded significant protection. In contrast, the NMDA receptor antagonists dizocilpine (MK-801) and memantine did not prevent cell death. Of the diuretics tested, furosemide was protective, whereas the other two anion exchanger inhibitors, L-644,711 [(*R*)-(+)-(5,6-dichloro2,3,9,9*a*-tetrahydro 3-oxo-9*a*-propyl-1*H*-fluoren-7-yl)oxy acetic acid] and bumetanide, were ineffective. In vitro comparison of these diuretics indicated that furosemide is also a potent antioxidant, whereas the nonprotective diuretics are not. The lack of efficacy of L-644,711 and bumetanide suggests that the antioxidant rather than the diuretic properties of furosemide contribute most critically to its efficacy in reversing ethanol-induced neurotoxicity in vitro, in our model. This study provides the first demonstration of CBD as an in vivo neuroprotectant and shows the efficacy of lipophilic antioxidants in preventing binge ethanol-induced brain injury.

Alcohol is the world's most widely used psychoactive drug, but chronic, excessive alcohol consumption leads to permanent organ damage or death. Alcohol-induced brain damage produces some of the most insidious effects of alcoholism, including cognitive deficits such as learning and memory impairment (Pfefferbaum et al., 1998; White, 2003). The pattern of alcohol consumption is an important predictor of brain damage with episodic or binge drinkers (defined as those who consume four to five or more drinks in a row) being the most

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vulnerable group (Hunt, 1993). In the United States, about 40% of college students and a high percentage of older adult alcoholics fit this definition of binge drinking (Wechsler et al., 2002).

The rat model of alcohol consumption used in the current study is designed to mimic a single cycle of binge drinking in human alcoholics. Binge ethanol consumption patterns in the rodent have been demonstrated to negatively impact on the ability of the rat to learn new information, not unlike memory difficulties seen in human alcoholics. This is consistent with the observation that binge ethanol consumption causes observable neuronal cell loss, measured by agyrophilic profiles, which is especially prominent in entorhinal cortex and hippocampus, two regions known to be involved in memory and cognition (Collins et al., 1996; Obernier et al., 2002a). The mechanism behind ethanol-induced selective neuronal damage is not well understood, but several explanations have been proposed. These include excitotoxicity associated with excessive neurotransmitter release, oxidative stress leading to free radical damage (Eskay et al., 1995; Crews et al., 2004), and edema caused by alterations in cellular control of ion transport (Collins et al., 1998).

Chronic ethanol consumption leads to increases in the density of NMDA receptor expression and subsequently the size of evoked calcium responses (Hu and Ticku, 1995). The non-competitive NMDA receptor antagonist dizocilpine (MK-801) has been shown to prevent alcohol withdrawal seizures (Grant et al., 1992) and has been previously examined as a treatment for alcohol-induced brain injury. Unfortunately, MK-801 treatment failed to ameliorate ethanol-induced neuronal damage (Collins et al., 1998; Corso et al., 1998), but this was possibly due to neurotoxic effects of MK-801 itself (Thomas et al., 2002). More recently, it has been proposed that low-affinity, activity-dependent NMDA receptor antagonists such as memantine may be able to protect against excitotoxicity, without also blocking the basal glutamatergic neurotransmission required for normal brain functioning (Lipton and Chen, 2004).

Ethanol may also injure the brain by increasing oxidative stress. Although the mechanisms behind oxidative stress are again not well understood, numerous studies have demonstrated that chronic ethanol consumption is accompanied by both oxidative damage to cellular proteins, lipids, and DNA (Mansouri et al., 2001; McDonough, 2003) and reduced levels of the endogenous antioxidants glutathione and superoxide dismutase (Reddy et al., 1999; Thirunavukkarasu et al., 2003). The role of oxidants in alcoholic tissue damage was well demonstrated by Mansouri et al. (2001) when they showed that acute ethanol administration in the mouse causes a loss of mitochondrial DNA in the brain and other tissues. Mansouri et al. (2001) also demonstrated that this damage can be prevented by either antioxidant administration or inhibiting ethanol metabolism, which suggests the involvement of mitochondrial dysfunction in alcohol-induced oxidative stress. Furthermore, Herrera et al. (2003) showed that chronic ethanol treatment in the rat results in a decline in hippocampal neurogenesis and that the antioxidant ebselen can prevent such decrease.

Brain edema has also been a demonstrated result of repeated intoxication and withdrawal cycles, which are the hallmark of binge alcohol consumption (Collins et al., 1998). Such alcoholics show brain edema during alcohol withdrawal, possibly due to over secretion of

vasopressin (Lambie, 1985). During cytotoxic brain edema, astrocytes and neuronal dendrites swell, which is believed to trigger a compensatory release of chloride ions and excitatory amino acids during the process of regulatory volume decrease (Aschner et al., 1999). The role for brain edema in alcohol-induced neuronal loss is supported by the efficacy of the diuretic furosemide, which has been shown to prevent such neurotoxicity (Collins et al., 1998).

In the current study, we use a rat model of binge alcohol consumption to determine the potential of cannabidiol (CBD) as a neuroprotectant against ethanol-induced neurotoxicity. We also compare the in vivo neuroprotectant effects of CBD to that provided by two categories of NMDA receptor antagonists, two other lipophilic antioxidants, and three diuretics, to determine the mechanism by which CBD protects against binge alcohol-induced damage.

Materials and Methods

Materials

Cannabidiol and reagents other than those specifically listed below were purchased from Sigma Chemical Co. (St. Louis, MO). Alkamuls EL-620 was a gift from Rhodia (Cranberry, NJ). Dihydrorhodamine was obtained from Molecular Probes (Eugene, OR). Hydrogen peroxide, tetraethylammonium chloride, ferric citrate, and sodium dithionite were all purchased from Aldrich Chemical Co. (Milwaukee, WI). L-644,711 was a gift from Merck (Somerset, NJ).

Solution Preparation for in Vivo Studies

Cannabidiol, butylated hydroxytoluene (BHT), and α-tocopherol (TOC) were administered in vivo using a saline vehicle containing 30% ethoxylated castor oil (Alkamuls EL-620) and 3% ethanol. Cannabidiol and BHT were first solubilized in ethanol and then mixed with Alkamuls EL-620. Since TOC (Sigma Chemical Co.) was supplied as a concentrate in corn oil, ethanol and Alkamuls were added to the TOC to achieve a similar preparation as described for CBD and BHT. Each preparation was diluted [1:1 (v/v)] with saline immediately before administration as a 2-ml i.p. bolus, which supplied either 20 or 40 mg/kg CBD, 40 mg/kg BHT, or 80 mg/kg TOC/day.

Furosemide was prepared by dissolving in 0.1 N NaOH, and the pH was adjusted to 7.3 with 0.1 N HCl. Then, 800 μl/kg of this 4.2 mg/ml solution was injected (s.c.) into rats every 8 h to achieve a dose of 10 mg/kg/day. Bumetanide was dissolved in ethanol and then diluted with PBS (0.2% ethanol in PBS) to a concentration of 0.9 mg/ml and given 400 μl/kg (s.c.) every 8 h to a total of 1 mg/kg/rat each day. L-644,711 was dissolved in PBS at 8.4 mg/ml and was injected 800 μl/kg (s.c.) every 8 h to give 20 mg/kg/day.

MK-801, nimodipine, and memantine were suspended in physiological saline and given s.c. every 4 h in a volume of 100 μl. MK-801 was given at 0.02 and 0.6 mg/kg/day, nimodipine at 6 mg/kg/day, and memantine 30 mg/kg/day.

Cyclic Voltammetry

Cyclic voltammetry was performed with a potentiostat/galvanostat (model 273/PAR 270 software; EG&G Princeton Applied Research, Princeton, NJ). The working electrode was a glassy carbon disk with a platinum counter electrode and silver/silver chloride reference. Tetraethylammonium chloride in 0.1 M acetonitrile was used as an electrolyte. Cyclic voltammetry scans were done from 0 to +1.8 V at a scan rate of 100 mV/s. All oxidation potentials are reported versus Ag/AgCl.

Iron-Catalyzed Dihydrorhodamine Oxidation (Fenton Reaction)

Antioxidant activities of test compounds were evaluated by their ability to prevent oxidation of dihydrorhodamine to the fluorescent compound rhodamine. Test compounds in a 50:50 water/acetonitrile (v/v) solution were incubated with 50 μM dihydrorhodamine for 5 min in the presence of 1 mM hydrogen peroxide and an iron catalyst (10 μM dithionite-reduced ferrous citrate). After this time, dihydrorhodamine protection was assessed by spectrofluorometry (excitation, 500 nm; emission, 570 nm).

Animals

Male Sprague-Dawley rats (Taconic Farms, Rockville, MD), weighing approximately 250 g, were maintained under a 12-h light/dark cycle (lights on 6:00 AM and off at 6:00 PM) with "Zeigler" (Gardners, PA) rat chow and water available ad libitum. For 7 days before surgery, all animals were group housed, but they were individually housed after implantation of chronic indwelling gastric cannula. All surgical procedures were performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Rats were anesthetized with ketamine hydrochloride/xylazine (80:10 mg/kg i.p.). Hair along the abdomen and nape of the neck was clipped, and the skin was swabbed with Betadine. A 3-cm incision was made through the skin adjacent to the stomach, and a 1-cm incision was made through the gastric musculature. A flared 10-cm piece of medical grade silicone tubing (0.095 o.d.; New Age Industries, Southampton, PA) was inserted into the gastric fundus, and the gastric musculature was sutured closed around it. The silicone tubing was inserted under the skin to the nape of the neck and connected to an L-shaped 5-cm hypodermic stainless steel tube (15 gauge; Small Parts, Miami lakes, FL) woven into a 2-cm square of Dacron mesh pad. The Dacron pad was sutured to the musculature and skin at the neck and the incision closed with 3.0 silk suture (Ethicon, Piscataway, NJ). The gastric cannula was flushed with 0.9% saline and capped. A single subcutaneous injection of buprenorphine (100 μg/kg) was given for postsurgical analgesia, along with i.p. antibiotics gentamicin (8.5 mg/kg) and ampicillin (70 mg/kg). Animals were placed on a heating pad until they became ambulatory, at which time they were returned to their cages and fed ad libitum.

Alcohol Administration Procedure

Seven days after cannula implantation, all rats were given ad libitum access to alcohol-free liquid diet for 3 days formulated to provide 16.9% of calories as protein, 59.2% carbohydrate, and 23.9% fat (Research Diets Inc., Allentown, NJ). The morning of the next day, alcohol administration was begun (day 1 of the 4-day binge). Beginning at lights on, all

rats were given 12 ml of liquid diet via gastric cannula every 8 h (6:00 AM, 2:00 PM, and 10:00 PM). In the ethanol-treated animals, the 12 ml of liquid diet was modified to contain 10 to 12% less calories from carbohydrate, which was replaced with an equal caloric quantity of ethanol. Rats were rated for level of intoxication at the time of each ethanol feeding and given the appropriate ethanol dose, as per the Majchrowicz procedure (Majchrowicz, 1975) for 4 days. Diuretics were administered s.c. three times daily, and glutamatergic blockers were administered s.c. six times daily for the 4-day ethanol treatment protocol. Cannabidiol and other antioxidants were administered i.p. twice a day (10:00 AM and 10:00 PM) on alcohol treatment days 2–4, since neurodegeneration has not been seen before 3 or 4 days of binge alcohol administration. All potential neuroprotectants tested were administered in a double-blinded manner. On the morning of the fifth day animals were deeply anesthetized with an i.p. injection of ketamine hydrochloride and xylazine (80:10 mg/kg) and transcardially perfused via gravity flow. Blood was cleared from the animal's vasculature with 200 ml of wash solution (0.8% sodium chloride, 0.4% dextrose, 0.8% sucrose, 0.5% sodium nitrite, 0.023% calcium chloride, and 0.034% sodium cacodylate) followed by 250 ml of fixative solution (4% sucrose, 4% paraformaldehyde, and 1.43% cacodylic acid).

Biochemical Procedures

Blood alcohol levels (BALs) were monitored each day by a tail bleed, taken 2 h after initial daily ethanol administration (8:00 AM), and BALs were determined using a standard alcohol dehydrogenase-based diagnostic kit (Sigma Chemical Co.).

Brain Preparation

Neurodegeneration was assessed using the amino-cupric-silver technique of De Olmos as performed by Switzer's Neuroscience Associates group (de Olmos et al., 1994; Switzer, 2000). In short, brains were removed from the skulls 24 h after perfusion and immersed in a solution containing 20% glycerol and 2% dimethyl sulfoxide to prevent freeze artifacts and then embedded in a gelatin matrix in groups of 16. The brain matrices were then sliced in the coronal plane by sliding microtome (40 μm in thickness). Every eighth section was then stained.

Staining Procedure

Briefly, sections were rinsed three times in deionized water and then placed for 4 days in an aqueous mixture of silver and copper nitrate, pyridine, and ethanol. Sections were then transferred through acetone, a diammine-silver solution, reduced in a weak formaldehyde solution, and bleached in a preparation of potassium ferricyanide and sodium borate (to remove unreduced silver). Sections were then mounted, dried, and counter stained with neutral red.

Quantification

Coronal sections from the ventral hippocampus, both left and right side (2 each side), were analyzed for degeneration counts at 6.00 and 6.32 mm posterior to bregma (Paxinos and Watson, 1986). Degenerating cells were defined as dark, argyrophilic neurons with dendrites

clearly visible. Dark objects not clearly identified as neurons were not counted. The number of counts was determined from two consecutive circular microscope fields at 20× magnification for each tissue section. Data for hippocampal degeneration are presented as counts per square millimeter by dividing total number of degenerating cells counted in eight microscope fields by the calculated area of the fields counted (1-mm-diameter counting field). Two sections from both the left and right side of the entorhinal cortex were also counted at 7.00 and 7.32 mm posterior to bregma, in one microscope field at 20× magnification.

Data Analysis

Data are reported as mean values ± standard error. Degeneration data were examined for significance (at $p < 0.05$) using the nonparametric Kruskal-Wallis test with Mann-Whitney *U* tests for pairwise comparisons.

Results

Analysis of rats fed 10 to 12% ethanol (9–12 g/kg/day) in a liquid diet confirmed that blood alcohol levels were maintained between 2.0 and 4.0 g/l on days 2 to 4 (Table 1). At the end of the experiment, silver staining of coronal brain slices prepared from ethanol-treated animals revealed a significant loss of neurons throughout the hippocampal cortical circuits of the brain (Fig. 1). The most affected regions included the olfactory bulb and dentate gyrus granular cell layer, as well as perirhinal, piriform, and entorhinal cortices. Silver-stained cells were quantified in the hippocampus and entorhinal cortex. When 40 mg/kg CBD was coadministered with ethanol on days 2 to 4 of the protocol, alcohol-induced cell death was reduced by approximately 60% ($p < 0.05$) in both hippocampal granular cells and the entorhinal cortical pyramidal cells (Fig. 1).

Since excitotoxicity has also been proposed as a potential mechanism underlying ethanol-induced neurodegeneration, the effects of two NMDA receptor antagonists and an L-type calcium channel blocker were examined. The NMDA receptor antagonists MK-801 (0.6 mg/kg/day) and memantine (30 mg/kg/day) were administered in six divided doses to both control rats and animals subjected to a binge ethanol paradigm (described above). Similarly, two other groups of animals were given nimodipine (6 mg/kg/day), an L-type calcium channel blocker, both with and without ethanol. Three-day average BALs measured 2 h after administration in ethanol-, ethanol plus MK-801-, ethanol plus memantine-, and ethanol plus nimodipine-treated rats were 2.66 ± 0.38, 2.24 ± 0.28, 2.68 ± 0.22, and 2.42 ± 0.36 g/l, respectively, and they did not differ across groups. Figures 2 and 3 demonstrate that argyrophilic staining in both the hippocampus and entorhinal cortex of ethanol-treated animals was actually increased by the presence of 0.6 mg/kg/day MK-801. Furthermore, staining of piriform cortical (not shown) and hippocampal granule cells was evident with MK-801 treatment even in the absence of alcohol (Fig. 2A). Due to the apparent toxicity of this dose of MK-801, a second experiment was performed using a 30-fold lower dose of MK-801 (0.02 mg/kg/day) in conjunction with the ethanol treatment. This dose of MK-801 was found to be without neurotoxic consequences when given in the absence of ethanol, but it was again found to exacerbate the alcohol-induced damage in the entorhinal cortex (Fig.

3C). At 0.02 mg/kg/day dizocilpine had neither a protective nor toxic effect on the hippocampal dentate gyrus (Fig. 2C). Similarly, the low-affinity antagonist memantine and the L-type calcium channel blocker nimodipine neither exacerbated nor ameliorated damage in either the dentate gyrus or the entorhinal cortex.

To further explore the mechanism underlying CBD neuroprotection against ethanol neurotoxicity, its efficacy was compared with that of antioxidants BHT and TOC both in vitro and in vivo. The antioxidant properties of CBD were compared in vitro with BHT and TOC, by cyclic voltammetry, which measures the oxidation or reduction potential (*x*-axis) of a compound. The *y*-axis, or current, is a measure of the number of electrons per unit time, reflecting primarily diffusion of the electrochemical species to and from the electrode surface. Cannabidiol, like BHT, exhibited an irreversible oxidation potential of 1.4 V (Fig. 4A), whereas TOC had a higher, but reversible potential of 1.5 V. By this criterion, CBD, BHT, and tocopherol are comparable antioxidants.

To examine whether the antioxidant property of CBD might account for the protection it provided in the binge drinking model, the effects of BHT and TOC were also examined using the same 4-day binge-alcohol administration model. Three-day average BALs for ethanol-, ethanol plus BHT-, and ethanol plus TOC-treated rats were 3.03 ± 0.18, 2.63 ± 0.27, and 2.65 ± 0.25 g/l, respectively, and they were not statistically different. Both compounds (BHT at 40 mg/kg and TOC at 80 mg/kg) significantly reduced neuronal loss in the hippocampus and entorhinal cortex to a similar degree to that seen with CBD (Fig. 4B), a result consistent with the hypothesis that CBD protects due to its antioxidant properties.

A previous binge alcohol administration study indicated that the diuretic furosemide, which acts by inhibiting both Cl^-/HCO^-_3 anion exchange and $Na^+/K^+/2Cl^-$ cotransport, also protects against alcohol-induced neurotoxicity (Collins et al., 1998). Although the mechanism by which furosemide protected was not confirmed, it was suggested that diuretics might protect by reducing alcohol-induced brain edema. To examine this hypothesis, furosemide and two other diuretics were compared in vivo. As with furosemide, bumetanide is a loop diuretic that inhibits $Na^+/K^+/2Cl^-$ cotransport, whereas L-644,711 is a modified loop diuretic that inhibits Cl^-/HCO^-_3 anion exchange. Three day average BALs for ethanol-, ethanol plus furosemide-, ethanol plus bumetanide-, and ethanol plus L-644,711-treated rats were 3.17 ± 0.33, 2.68 ± 0.33, 3.41 ± 0.17, and 3.17 ± 0.29 g/l, respectively, and they did not differ statistically. As shown by Collins, 10 mg/kg furosemide significantly reduced cell loss in the entorhinal cortical neurons to a degree similar to that observed with CBD or other antioxidant treatments (Fig. 5, A and B). In contrast, neither 1 mg/kg bumetanide nor 20 mg/kg L-644,711 provided any significant protection against alcohol-induced neurotoxicity (Fig. 5, A and B).

The inconsistent protection observed with the three diuretics suggested that some other feature of furosemide might explain its protective properties against alcohol-induced injury. Since antioxidants provided significant protection in this model, the oxidation potentials of the three tested diuretics were examined by cyclic voltammetry. The results revealed that the oxidation potential of furosemide and bumetanide were similar to that of the antioxidants described above (compare Figs. 4A and 5C). In contrast, L-644,711 showed no appreciable

ability to be oxidized and therefore would not have antioxidant properties. Since bumetanide had similar oxidation potential to the other protective compounds and yet failed to protect against alcohol-induced neurotoxicity, a second, antioxidant assay was performed. In the Fenton reaction assay, varying concentrations of a test compound are examined for their ability to prevent dihydrorhodamine oxidation by oxygen free radicals (generated by ferrous catalysis of hydrogen peroxide). This assay helps predict which of those compounds with antioxidant activity are likely to be effective antioxidants in a biological context, e.g., when antioxidant activity requires free-radical scavenging capability. Furosemide, as well as the antioxidants CBD and BHT, inhibited dihydrorhodamine oxidation with a similar relative EC_{50} of approximately 1 mM (the exact EC_{50} value is dependent on the amount of oxidant present). Despite the oxidation potential of bumetanide, as measured by cyclic voltammetry, it did not prevent oxidation of dihydrorhodamine (Fig. 5D). These in vitro antioxidant assays strongly suggest that furosemide is a biological antioxidant, unlike bumetanide or L-644,711. Together, the data presented in this study demonstrate that antioxidants, exhibiting both antioxidant potential by cyclic voltammetry and capable of inhibiting the oxidation of dihydrorhodamine in the Fenton reaction, protect against alcohol-induced brain injury, whereas the inconsistent protection observed with diuretics suggests that furosemide protection is at least partly a function of its antioxidative properties.

Discussion

Rats exposed to a 4-day binge ethanol treatment had significant cellular damage in the olfactory bulb, perirhinal, piriform, and entorhinal cortices, and the dentate gyrus granular cell layer, in agreement with previous reports (Collins et al., 1996; Obernier et al., 2002a). Since alcohol circulates freely through the whole brain, rather than selectively to these regions, it seemed most likely that damage to hippocampus and entorhinal cortex by ethanol reflects differential susceptibility of these neuronal populations to ethanol-induced damage, similar to ischemia-induced neuronal cell loss (Back et al., 2004).

Quantitative measurements of selected putative neuroprotectants were examined in the hippocampus and entorhinal cortex, areas with intense silver staining. It has previously been demonstrated that this silver staining technique identifies cells irreversibly committed to the cell death pathway (Switzer, 2000). Damage to these brain areas is particularly important because of their involvement in memory formation and recall. The selective vulnerability of the hippocampus and entorhinal cortex may therefore lead to significant behavioral sequela in binge drinkers impacting cognitive function and independence in performing activities of daily life. Rats demonstrate significant impairment in learning ability (Morris water maze test) 5 days after binge ethanol consumption (Obernier et al., 2002b) spending more time than controls in a previously trained quadrant during reversal training, demonstrating perseveration of an incorrect response not unlike that reported in human alcoholics (Lyvers and Maltzman, 1991).

We demonstrate here that CBD, a nonpyschoactive component of marijuana, substantially limits neuronal damage to hippocampal and entorhinal cortical brain regions when administered concurrently with alcohol in the rat binge alcohol model. CBD has been shown to prevent damage associated with glutamate toxicity in cortical neuron cultures and to have

antioxidant properties (Hampson et al., 1998). We therefore examined both excitotoxic and antioxidant pathways for their neuroprotective ability. We attempted to inhibit excitotoxic damage in vivo through NMDA receptor blockade using both high- and low-affinity compounds. The high-affinity antagonist MK-801 protects organotypic slice cultures exposed to NMDA (Pringle et al., 2000) and eliminates alcohol withdrawal seizures (Grant et al., 1992) and accompanying deficits in reversal learning (Thomas et al., 2002). Previous work, however (Collins et al., 1998; Corso et al., 1998), failed to show neuroprotection against binge ethanol-induced neurodegeneration in rats given 1 or 2 mg/kg/day MK-801. We tested MK-801 at a dose (0.6 mg/kg/day) reported to be protective against the teratogenic affects of ethanol withdrawal (Thomas et al., 2002). In accord with results of Collins et al. (1998), rats treated with 0.6 mg/kg/day MK-801 demonstrated dramatic enhancement of, rather than protection from, ethanol-induced cellular damage in hippocampal and entorhinal cortical regions. In fact, MK-801 was toxic to hippocampal granule cells even in the absence of ethanol. Corso et al. (1998) reported no damage with 1 mg/kg/day MK-801 in the absence of ethanol with a single daily injection. The difference in these two reports may be the MK-801 administration frequency (half-life in rats 90 min; Schwartz and Wasterlain, 1991). An indwelling minipump (2 mg/kg/day) resulted in extensive damage, but a single injection (1 mg/kg/day) caused no damage. In our experiment, 0.6 mg/kg/day resulted in damage (six divided doses), suggesting that frequency and dosage contribute to the in vivo neurotoxicity of MK-801.

Administration of substantially less MK-801 (0.02 mg/kg/day) was without neurotoxic effects itself, whereas significantly exacerbating ethanol neurotoxicity in the entorhinal cortex. Thus, high-affinity NMDA receptor antagonists, although predictive of decreased glutamate-induced toxicity in cell culture, seem to have no therapeutic value in the binge ethanol model.

A new class of low-affinity antagonists have been developed with rapid on/off receptor-binding kinetics (Lipton and Chen, 2004). Memantine inhibits the development of alcohol dependence, evidenced by abrogation of audiogenic seizures (Kotlinska, 2001), and prevents ethanol-induced cognitive impairment in the rat (Lukoyanov and Paula-Barbosa, 2001). Memantine, however, did not affect the extent of neuronal damage in these studies. Blockade of L-type calcium channels also had no beneficial effects on alcohol-induced cell damage. Thus, it seems unlikely that the neuroprotection afforded by CBD occurs through inhibition of the excitotoxic cascade initiated by glutamate release. Rather, the present experiments confirm our previous cell culture findings, demonstrating that CBD neuroprotection against glutamate-induced neurotoxicity likely involves redox events downstream of NMDA receptor occupancy, engagement, and subsequent calcium influx (Hampson et al., 1998).

We compared the neuroprotective effects of CBD with two other common antioxidants, TOC and BHT. All three compounds reduced more than 50% of the argyrophilia after binge ethanol treatment in brain regions examined. Acute and chronic ethanol administration has demonstrated increases in reactive oxygen species such as malondialdehyde and reductions in superoxide dismutase and glutathione reductase antioxidant enzyme activity (Reddy et al., 1999; Thirunavukkarasu et al., 2003). Administration of the endogenous antioxidant

melatonin, or quercetin, a flavonoid antioxidant, reverses brain lipid peroxidation and ameliorates memory retention deficits in chronically ethanol-treated mice (Raghavendra and Kulkarni, 2001; Singh et al., 2003). Because the brain contains a high concentration of polyunsaturated fatty acids and low levels of antioxidants (Sun and Sun, 2001), it may be particularly vulnerable to ethanol-induced oxidative stress. We show here that antioxidants prevent the cell death associated with binge ethanol consumption.

The 4-day binge ethanol model has been used to demonstrate not only neurodegeneration but also decreased proliferation and survival of nascent neural progenitors (Nixon and Crews, 2002). Ebselen, an antioxidant, completely reversed the decline in neuron generation in the granule cell layer after chronic ethanol treatment (Herrera et al., 2003). Thus, antioxidants seem to enhance neuronal replacement as well as prevent neuronal death after ethanol insult.

Previously, the compound furosemide was found neuroprotective in a similar binge model of alcohol-induced brain damage (Collins et al., 1998). It was hypothesized that the protective ability of furosemide derived from its diuretic properties, preventing compression-related trauma associated with fluid imbalance and cellular swelling due to high ethanol concentrations. We demonstrate here that compounds with similar abilities to inhibit anion exchange (bumetanide and L-644,711), one of which is neuroprotective in a mechanical trauma model (Kimelberg et al., 1989), did not prevent the alcohol-associated damage in the hippocampus and entorhinal cortex. Examination of furosemide and bumetanide revealed oxidation potentials similar to those of BHT and TOC. Only furosemide, however, effectively prevented oxidation in a Fenton reaction, revealing superior potential as a biological antioxidant.

The mechanisms by which alcohol induces neuronal oxidative damage are not known and could potentially include acetaldehyde-derived alkaloidal metabolite generation or inflammatory mechanisms. TNFα, a potent cytokine inducer of neutrophil infiltration and generator of oxidative species in microglia and macrophages, has a prominent role in alcohol-induced liver disease, but its role in alcohol-induced neurotoxicity is unknown. CBD has been shown to decrease TNFα release in a murine collagen-induced arthritis model (Malfait et al., 2000), and BHT can block TNFα activation of nuclear factor-κB (Zou and Crews, 2005), demonstrating that antioxidants can decrease inflammation. Oxidative stress and inflammation are likely linked and may be difficult to tease apart. Our findings here suggest that antioxidant properties rather than diuresis alone contribute most critically to protection against alcohol-induced neurotoxicity and that although individual measures of antioxidant activity may predict antioxidant potential, predictive assessment of antioxidant activity in vivo may be complex.

It is perhaps unsurprising that biological antioxidant activity in vivo may not be predictable based solely on electrochemical potential (or indeed any single in vitro parameter of redox activity). The biological compartment into which the compound partitions, the reactive species with which it interacts, and the oxidized species generated all likely effect biological antioxidant efficacy. In addition, the biochemical profile of oxidative stress is likely to differ depending on the pathophysiological stress, including the presence of infiltrating

inflammatory cells, compromised vascular perfusion, and altered glial and microglial cell function. Imparting antioxidant potential may therefore require optimization of electrochemical properties, rather than maximization, in a drug series, depending on initial lipophilicity, tissue distribution, cellular compartmentalization, and interaction with multiple cellular and extracellular oxidants and pathogenic oxidative pathways.

Although neuroprotection is potentially afforded by mechanisms in addition to antioxidation, the robust effects observed here for CBD, TOC, BHT, and furosemide suggest that designing general antioxidant properties into drugs with other primary mechanisms of action, or even the use of combination therapy may be beneficial in decreasing drug toxicity and enhancing drug efficacy. Assuming antioxidant properties as a primary mechanism of action opens the possibility for use of chemical methods as predictors of therapeutic outcome. Cyclic voltammetry is indicative of a substrate's capacity to yield electrons through an outer sphere electron transfer mechanism, whereas the Fenton reaction accounts for other mechanisms important in preventing oxidative tissue injury such as scavenging properties or interaction with metal catalysts. We submit that cyclic voltammetry is an "initial screen" for possible consideration as a biological antioxidant, whereas activity in the Fenton assay can "screen out" compounds before testing in vivo. The ability to couple convenient in vitro assays for antioxidant activity with an in vivo measurement of neuroprotection may be useful in iterative screening for neuroprotective compounds in a variety of disease states in which neurodegeneration occurs.

ABBREVIATIONS

NMDA	*N*-methyl D-aspartate
MK-801	dizocilpine
CBD	cannabidiol
L-644	711, (*R*)-(+)-(5,6-dichloro2,3,9,9*a*-tetrahydro 3-oxo-9*a*-propyl-1*H*-fluoren-7-yl)oxy acetic acid
Alkamuls EL-620	polyoxyethylene 30 castor oil
BHT	butylated hydroxytoluene
TOC	*α*-tocopherol
PBS	phosphate-buffered saline
BAL	blood alcohol level
TNF	tumor necrosis factor

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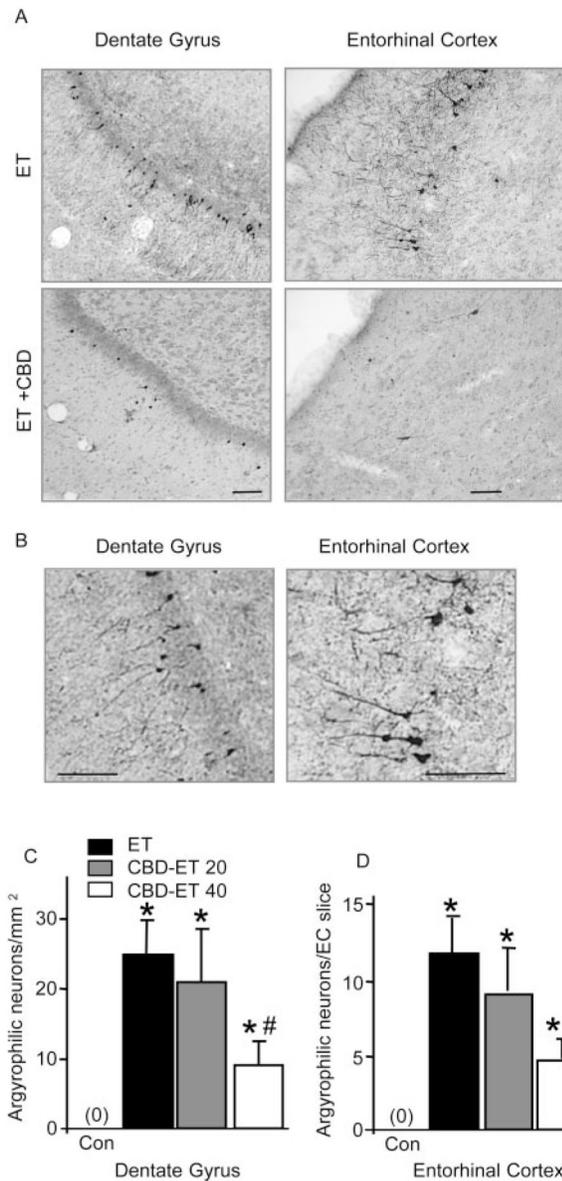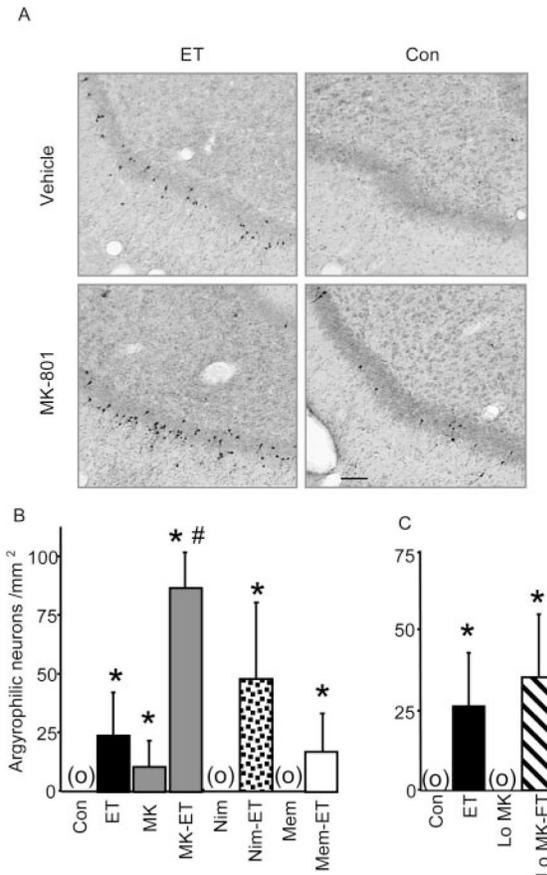
Fig. 1.
Effect of CBD on ethanol-induced neurotoxicity in rats. Gastric-cannulated rats were randomly divided into six groups: pair-fed controls (Con; $n = 5$), ethanol (ET; $n = 9$), cannabidiol 20 mg/kg (CBD20; $n = 6$), cannabidiol 20 mg/kg + ET (CBD-ET 20; $n = 8$), CBD 40 mg/kg (CBD40; $n = 6$), and CBD 40 mg/kg + ET (CBD-ET 40; $n = 8$) and given ET three times daily in a binge ethanol model as described under *Materials and Methods*. CBD was given i.p. twice daily on days 2 to 4. A, photomicrographs of damage in hippocampus (left) and entorhinal cortex (right) after 4 days of binge ethanol administration with either concurrent vehicle (top) or CBD 40 mg/kg/day (bottom) treatment. Cells were stained by de Olmos silver staining method as described under *Materials and Methods*. B, enlarged photomicrographs of degeneration in dentate gyrus (left) and entorhinal cortex (right) of ethanol-treated rats depicting greater detail in the silver staining. Graphical

representation of argyrophilic cell quantification in dentate gyrus (C) and entorhinal cortex (D). Silver staining was counted as described under *Materials and Methods*. Data represent mean values ± S.E.M. *, significant difference from Con, $p < 0.05$; #, significant difference from ET, $p < 0.05$, Mann-Whitney *U* pairwise comparisons. Data not shown for CBD-treated controls; no degeneration was found. Scale bar, 100 μm.

Fig. 2.

Effect of NMDA receptor blockade on ethanol-induced neurotoxicity in rat hippocampus. Gastric-cannulated rats were randomly divided into eight groups: pair-fed controls (Con; $n = 5$), ethanol (ET; $n = 6$), MK-801 (MK; $n = 6$), MK + ET (MK-ET; $n = 8$), nimodipine (Nim; $n = 6$), Nim + ET (Nim-ET; $n = 8$), memantine (Mem; $n = 8$), and Mem + ET (Mem-ET; $n = 11$) and given ET three times daily in a binge ethanol model as described under *Materials and Methods*. Potential neuroprotectants were given s.c. in six divided doses with daily totals for MK-801 (MK and MK-ET) at 0.6 mg/kg/day, nimodipine (Nim and Nim-ET) at 6 mg/kg/day, and memantine (Mem and Mem-ET) at 30 mg/kg/day just before ET administration and 4 h thereafter. A, photomicrographs of hippocampus granule cell layer after 4 days of binge ethanol administration (ET, left) compared with control diet (Con, right) treated with either vehicle (top) or MK-801 0.6 mg/kg/day (bottom). Cells were stained by De Olmos silver staining method as described under *Materials and Methods*. B, graphical representation of argyrophilic cell counting. Data represent mean values ± S.E.M. *, significant difference from Con, $p < 0.05$; #, significant difference from ET, $p < 0.05$, Mann-Whitney *U* pairwise comparisons. C, a second experiment was run as in B. Rats were divided into four groups: Con ($n = 6$), ET ($n = 8$), Lo MK ($n = 8$), and Lo MK-ET ($n = 11$) and given ET and MK-801 as described above using 0.02 mg/kg/day MK-801 in six divided doses. *, significant difference from Con, $p < 0.05$; Mann-Whitney *U* pairwise comparisons. Scale bar, 100 μm.
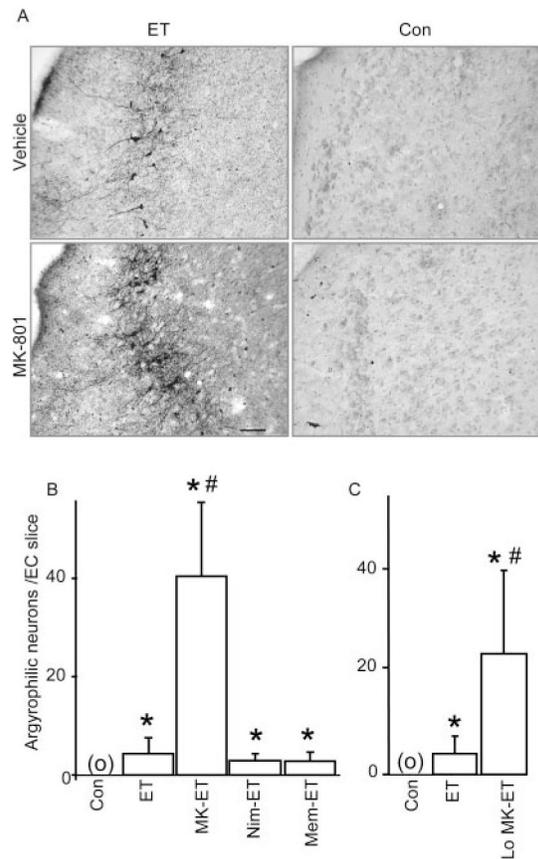
Fig. 3.
Effect of NMDA receptor blockade on ethanol-induced neurotoxicity in rat entorhinal cortex. Experimental conditions as described in Fig. 2. A, photomicrographs of entorhinal cortex after 4 days of binge ethanol administration (ET, left) compared with control diet (Con, right) treated with either vehicle (top) or MK-801 0.6 mg/kg/day (bottom). Cells were stained by De Olmos silver staining method as described under *Materials and Methods*. B, graphical representation of argyrophilic cell counting. Con ($n = 5$), ET ($n = 6$), MK-801 (MK; $n = 6$), MK + ET (MK-ET; $n = 8$), nimodipine (Nim; $n = 6$), Nim + ET (Nim-ET; $n = 8$), memantine (Mem; $n = 8$), and Mem + ET (Mem-ET; $n = 11$). Data represent mean values ± S.E.M. *, significant difference from Con, $p < 0.05$; #, significant difference from ET, $p < 0.05$, Mann-Whitney U pairwise comparisons. C, a second experiment was run as in Fig. 2B, using 0.02 mg/kg/day MK-801 in addition to, or apart from, ethanol treatment. Con ($n = 6$), ET ($n = 8$), Lo MK ($n = 8$), and Lo MK-ET ($n = 11$). Data represent mean values ± S.E.M. *, significant difference from Con, $p < 0.05$; #, significant difference from ET, $p < 0.05$, Mann-Whitney U pairwise comparisons. Scale bar, 200 μm.
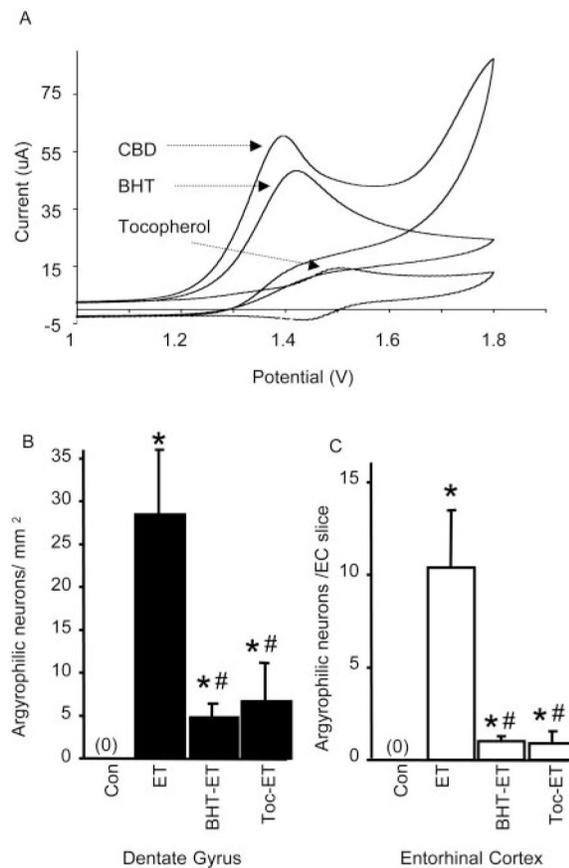
Fig. 4.
Comparison of the antioxidative capacities of CBD, BHT, and a-tocopherol and their neuroprotective effects. A, oxidation profiles of 1 mM CBD, BHT, and a-tocopherol when subjected to cyclic voltammetry. Data represent mean values ± S.D. from six replicates. Quantification of degeneration in rat hippocampus (B) and entorhinal cortex (C) after 4-day binge ethanol administration as described in Fig. 1. Rats were divided into six groups: Con ($n = 6$), ET ($n = 8$), BHT ($n = 6$), BHT + ET (BHT-ET; $n = 10$), tocopherol (TOC; $n = 6$), and TOC + ET (TOC-ET; $n = 7$). BHT and TOC were given i.p. twice daily on days 2 to 4. BHT was used at a dose of 40 mg/kg, whereas TOC was administered at 80 mg/kg. Data represent mean values ± S.E.M. *, significant difference from Con, $p < 0.05$; #, significant difference from ET, $p < 0.05$, Mann-Whitney U pairwise comparisons. Data from BHT and TOC without ET treatment not shown; no degeneration seen.
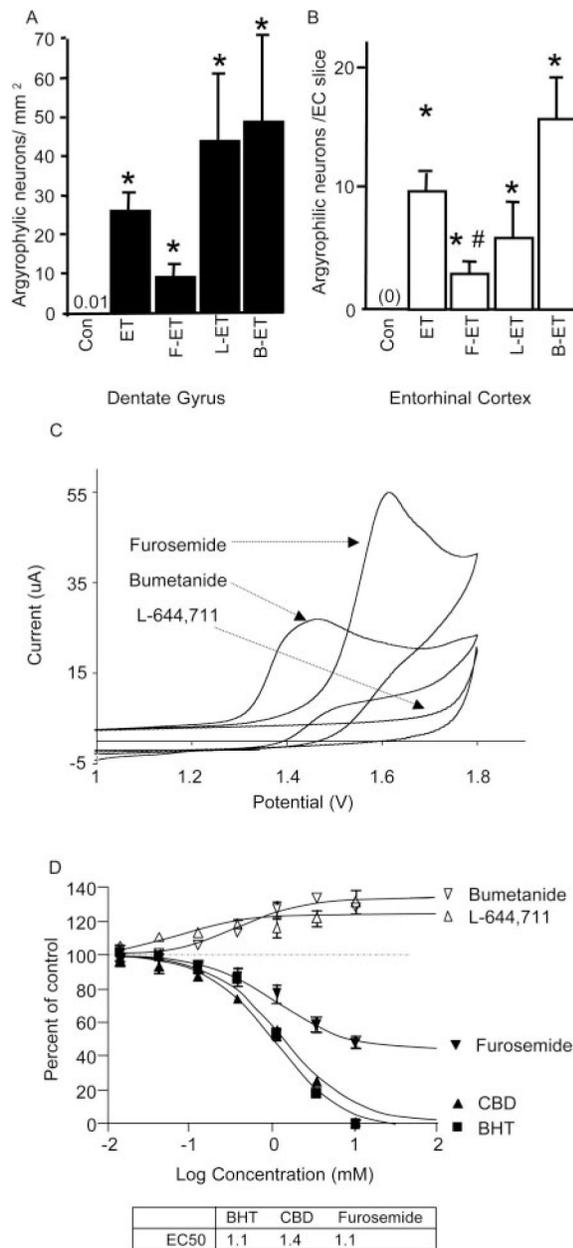
Fig. 5.
Comparison of furosemide and other compounds with similar pharmacological properties. A, in vivo comparison of diuretics as neuroprotectants in the hippocampus during binge ethanol consumption. Rats were divided into eight groups: Con ($n = 6$), ET ($n = 8$), furosemide (F, $n = 6$), F + ET (F-ET; $n = 5$), bumetanide (B; $n = 6$), B + ET (B-ET; $n = 7$), L-644,711 (L; $n = 6$), and L + ET (L-ET; $n = 9$) and were given intragastric ethanol for 4 days as in Fig. 1. Furosemide was given 10 mg/kg, bumetanide 1 mg/kg, and L-644,711 1 mg/kg. Data represent mean values ± S.E.M. *, significant difference from Con, $p < 0.05$; Mann-Whitney U pairwise comparisons. Data from F, B, and L without ET treatment not shown; no degeneration seen. B, quantification of degeneration in entorhinal cortex.

Experimental conditions same as in A. Data represent mean values ± S.E.M. *, significant difference from Con, $p < 0.05$; #, significant difference from ET, $p < 0.05$, Mann-Whitney U pairwise comparisons. Data from F, B, and L without ET treatment not shown; no degeneration seen. C, oxidation profile of 1 mM furosemide, bumetanide, and L-644,711 subjected to cyclic voltammetry. Data represent mean values ± S.D. from six replicates. D, antioxidative properties of furosemide were compared with two other blood-brain barrier-permeable anion exchange inhibitors and the antioxidants CBD and BHT using a Fenton reaction system based on the oxidation of dihydrorhodamine by 1 mM hydrogen peroxide (with a 10 μM ferrous citrate catalyst). See *Materials and Methods* for experimental details. Data represent mean values ± S.D. from six replicates.

TABLE 1

Blood alcohol levels

Rats were tail bled 2 h after the first ethanol feeding of the day, and blood alcohol levels were quantified using a standard alcohol dehydrogenase-based diagnostic kit.

Treatment Group	Day 2	Day 3	Day 4	3-Day Average
			g/l	
ET	2.59 ± 0.31	3.45 ± 0.27	3.60 ± 0.19	3.19 ± 0.22
ET + CBD, 20 mg/kg	2.00 ± 0.37	3.65 ± 0.30	3.47 ± 0.25	2.99 ± 0.22
ET + CBD, 40 mg/kg	2.53 ± 0.25	3.05 ± 0.20	3.48 ± 0.27	3.03 ± 0.18

ET, ethanol-treated rats.