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Title: Comparison of cannabidiol, antioxidants and diuretics in reversing binge ethanol-induced neurotoxicity

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Running Title: Antioxidants ameliorate alcohol-induced neurodegeneration

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Abbreviations used: BAL blood alcohol level, CBD cannabidiol, BHT butylated hydroxytoluene, TOC α-tocopherol, EC entorhinal cortex, CNS central nervous system, NMDA n-methyl D-aspartate, i.p. intraperitoneally, s.c. subcutaneously, L-644,711 (R)-(+)-(5,6-dichloro2,3,9,9a-tetrahydro 3-oxo-9a-propyl-1H-fluoren-7-yl)oxy acetic acid, MK-801dizocilpine

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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, while NMDA receptor activity has been implicated in alcohol-withdrawal and excitoxic injury. As the non-psychoactive 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 fashion. Similarly, the common antioxidants butylated hydroxyltoluene and alpha 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 and bumetanide were ineffective. In vitro comparison of these diuretics indicated that furosemide is also a potent antioxidant while the non-protective 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.

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Introduction

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 4-5 or more drinks in a row) being the most vulnerable group (Hunt, 1993). In the U. S. 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).

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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. While 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 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

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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, in order to determine the mechanism by which CBD protects against binge alcohol-induced damage.

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Materials and Methods

Materials. Cannabidiol and reagents other than those specifically listed below were purchased from Sigma Chemicals, (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 (Milwaukee, WI). L-644,711 [(R)-(+)-(5,6-dichloro2,3,9,9a-tetrahydro 3-oxo-9a-propyl-1H-fluoren-7-yl)oxy acetic acid] was a gift from Merck (Somerset, NJ).

Solution preparation for in vivo studies. Cannabidiol, butylated hydroxyl toluene (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 Chemicals, St. Louis, MO) was supplied as a concentrate in corn oil, ethanol and Alkamuls were added to the TOC, in order to achieve a similar preparation as described for CBD and BHT. Each preparation was diluted (1:1, v/v) with saline immediately prior to 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 per day.

Furosemide was prepared by dissolving in 0.1 N NaOH, and then adjusting to pH 7.3 with 0.1 N HCl. 800 μl/kg of this 4.2 mg/ml solution was injected (s.c.) into rats every 8 hours in order to achieve a dose of 10 mg/kg/day. Bumetanide was dissolved in ethanol 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 hours to a total of 1mg/kg/rat each day. L-644,711 was

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dissolved in PBS at 8.4 mg/ml and was injected 800μl/kg (s.c.) every 8 hours to give 20 mg/kg/day.

Dizocilpine (MK-801), nimodipine and memantine were suspended in physiological saline and given s.c. every 4 hours in a volume of 100 ul. 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 an EG&G Princeton Applied Research potentiostat/galvanostat (Model 273 / PAR 270 software, Princeton, NJ). The working electrode was a glassy carbon disk with a platinum counter electrode and silver/silver chloride reference. Tetraethylammonium chloride in acetonitrile (0.1 M) was used as an electrolyte. Cyclic voltammetry scans were done from 0 to +1.8 V at scan rate of 100 mV per second. All oxidation potentials are reported versus Ag/AgCl.

Iron catalysed 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 spectrofluorimetry (excitation =500 nm, emission =570 nm).

Animals. Male Sprague-Dawley rats (Taconic Farms, Rockville, MD), weighing approximately 250g, were maintained under a 12 hour light/dark cycle (lights on 0600 hrs and off at 1800 hrs) with “Zeigler” (Gardners, PA) rat chow and water available *ad*

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libitum. For 7 days prior to surgery all animals were group housed, but were individually housed after implantation of chronic indwelling gastric cannulae. 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 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 (New Age Industries, Southampton, PA; 0.095 o.d.) 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 (Small Parts, Miami lakes, FL; 15 gage) woven into a 2 cm square of dakron mesh pad. The dakron 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 post-surgical analgesia, along with i.p. antibiotics gentamicin (8.5 mg/kg) and amipicillin (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 following 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 eight hours (0600, 1400, and 2200 hours). In the ethanol treated

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animals, the 12 ml of liquid diet was modified to contain 10-12% 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 four days. Diuretics were administered s.c. 3 times daily and glutamatergic blockers were administered s.c. 6 times daily for the 4-day ethanol treatment protocol. Cannabidiol and other antioxidants were administered i.p. twice a day (1000 and 2200 hours) on alcohol treatment days 2-4, since neurodegeneration has not been seen prior to 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 (BAL) were monitored each day by a tail bleed, taken 2 hours after initial daily ethanol administration (0800 hours) and BALs were determined using a standard alcohol dehydrogenase-based diagnostic kit (Sigma, St. Louis, MO).

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 hours after perfusion and immersed in a solution containing 20% glycerol and 2% dimethylsulfoxide to prevent freeze artifacts and then embedded in a gelatin matrix in

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groups of 16. The brain matrices were then sliced in the coronal plane by sliding microtome (40 μm thick). Every eighth section was then stained.

Staining procedure. Briefly, sections were rinsed 3 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 ea 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 20X magnification for each tissue section. Data for hippocampal degeneration are presented as counts per mm^2 by dividing total number of degenerating cells counted in 8 microscope fields by the calculated area of the fields counted (1mm 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 20X magnification.

Data analysis. Data are reported as mean values, plus and minus standard error. Degeneration data was examined for significance (at $p < 0.05$) using the nonparametric Kruskal Wallis test with Mann Whitney U tests for pair-wise comparisons.

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Results

Analysis of rats fed 10-12% ethanol (9-12 g/kg/day) in a liquid diet confirmed that blood alcohol levels were maintained between 2.0-4.0 g/L on days 2-4 (Table1). 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 (figure 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 co-administered with ethanol on days 2-4 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 (figure 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 per day), and memantine, (30 mg/kg/day), were administered in 6 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 hours 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 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

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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 (figure 2a). Due to the apparent toxicity of this dose of MK-801, a second experiment was performed using a thirty-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 was again found to exacerbate the alcohol-induced damage in the entorhinal cortex (figure 3c). At 0.02 mg/kg/day dizocilpine had neither a protective nor toxic effect on the hippocampal dentate gyrus (figure 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 (figure 4a), while TOC had a higher, but reversible potential of 1.5V. 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

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examined using the same four 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 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 (figure 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 $Na^+/K^+/2Cl^-$ co-transport), 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 $Na^+/K^+/2Cl^-$ co-transport, while 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 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 (figure 5a and 5b). In contrast neither 1 mg/kg bumetanide nor 20 mg/kg L-644,711 provided any significant protection against alcohol-induced neurotoxicity (figure 5a and 5b).

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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 figure 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 (figure 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

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alcohol-induced brain injury, while the inconsistent protection observed with diuretics suggests that furosemide protection is at least partly a function of its antioxidative properties.

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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 sequelae 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) five 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 non-pyschoactive component of marijuana, substantially limits neuronal damage to hippocampal and entorhinal cortical brain regions

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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 1mg/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 minutes (Schwartz and Wasterlain, 1991)). An indwelling mini pump (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 (6 divided doses), suggesting that frequency and dosage contributes to the *in vivo* neurotoxicity of MK-801.

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Administration of substantially less MK-801 (0.02 mg/kg/day) was without neurotoxic effects itself, while significantly exacerbating ethanol neurotoxicity in the entorhinal cortex. Thus, high affinity NMDA receptor antagonists, although predictive of decreased glutamate-induced toxicity in cell culture, appear 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 appears 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 involve 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 fifty percent of the argyrophilia following binge ethanol treatment in brain regions examined. Acute and chronic ethanol administration have 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.,

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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). As 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 following chronic ethanol treatment (Herrera et al., 2003). Thus, antioxidants appear to enhance neuronal replacement as well as prevent neuronal death following 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

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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. TNF-alpha, 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 NF-kB (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 while 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

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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, while 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, while activity in the Fenton assay can 'screen out' compounds prior to 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.

References

Aschner M, Allen JW, Kimelberg HK, LoPachin RM and Streit WJ (1999) Glial cells in neurotoxicity development. *Annu Rev Pharmacol Toxicol* **39**:151-173.

Back T, Hemmen T and Schuler OG (2004) Lesion evolution in cerebral ischemia. *J Neurol* **251**:388-397.

Collins MA, Corso TD and Neafsey EJ (1996) Neuronal degeneration in rat cerebrocortical and olfactory regions during subchronic "binge" intoxication with ethanol: possible explanation for olfactory deficits in alcoholics. *Alcohol Clin Exp Res* **20**:284-292.

Collins MA, Zou JY and Neafsey EJ (1998) Brain damage due to episodic alcohol exposure in vivo and in vitro: furosemide neuroprotection implicates edema-based mechanism. *Faseb J* **12**:221-230.

Corso TD, Mostafa HM, Collins MA and Neafsey EJ (1998) Brain neuronal degeneration caused by episodic alcohol intoxication in rats: effects of nimodipine, 6,7-dinitro-quinoxaline-2,3-dione, and MK-801. *Alcohol Clin Exp Res* **22**:217-224.

Crews FT, Collins MA, Dlugos C, Littleton J, Wilkins L, Neafsey EJ, Pentney R, Snell LD, Tabakoff B, Zou J and Noronha A (2004) Alcohol-induced neurodegeneration: when, where and why? *Alcohol Clin Exp Res* **28**:350-364.

de Olmos JS, Beltramino CA and de Olmos de Lorenzo S (1994) Use of an amino-cupric-silver technique for the detection of early and semiacute neuronal degeneration caused by neurotoxicants, hypoxia, and physical trauma. *Neurotoxicol Teratol* **16**:545-561.

JPET Fast Forward. Published on May 5, 2005 as DOI: 10.1124/jpet.105.085779
This article has not been copyedited and formatted. The final version may differ from this version.

Eskay RL, Chautard T, Torda T, Daoud RI and Hamelink C (1995) Alcohol, corticosteroids, energy utilization, and hippocampal endangerment. *Ann N Y Acad Sci* **771**:105-114.

Grant KA, Snell LD, Rogawski MA, Thurkauf A and Tabakoff B (1992) Comparison of the effects of the uncompetitive N-methyl-D-aspartate antagonist (+-)-5-aminocarbonyl-10,11-dihydro-5H-dibenzo[a,d] cyclohepten-5,10-imine (ADCI) with its structural analogs dizocilpine (MK-801) and carbamazepine on ethanol withdrawal seizures. *J Pharmacol Exp Ther* **260**:1017-1022.

Hampson AJ, Grimaldi M, Axelrod J and Wink D (1998) Cannabidiol and (-)Delta9-tetrahydrocannabinol are neuroprotective antioxidants. *Proc Natl Acad Sci U S A* **95**:8268-8273.

Herrera DG, Yague AG, Johnsen-Soriano S, Bosch-Morell F, Collado-Morente L, Muriach M, Romero FJ and Garcia-Verdugo JM (2003) Selective impairment of hippocampal neurogenesis by chronic alcoholism: protective effects of an antioxidant. *Proc Natl Acad Sci U S A* **100**:7919-7924.

Hu XJ and Ticku MK (1995) Chronic ethanol treatment upregulates the NMDA receptor function and binding in mammalian cortical neurons. *Brain Res Mol Brain Res* **30**:347-356.

Hunt WA (1993) Are binge drinkers more at risk of developing brain damage? *Alcohol* **10**:559-561.

Kimelberg HK, Rose JW, Barron KD, Waniewski RA and Cragoe EJ (1989) Astrocytic swelling in traumatic-hypoxic brain injury. Beneficial effects of an inhibitor of

JPET Fast Forward. Published on May 5, 2005 as DOI: 10.1124/jpet.105.085779
This article has not been copyedited and formatted. The final version may differ from this version.

anion exchange transport and glutamate uptake in glial cells. *Mol Chem Neuropathol* **11**:1-31.

Kotlinska J (2001) NMDA antagonists inhibit the development of ethanol dependence in rats. *Pol J Pharmacol* **53**:47-50.

Lambie DG (1985) Alcoholic brain damage and neurological symptoms of alcohol withdrawal--manifestations of overhydration. *Med Hypotheses* **16**:377-388.

Lipton SA and Chen HS (2004) Paradigm shift in neuroprotective drug development: clinically tolerated NMDA receptor inhibition by memantine. *Cell Death Differ* **11**:18-20.

Lukoyanov NV and Paula-Barbosa MM (2001) Memantine, but not dizocilpine, ameliorates cognitive deficits in adult rats withdrawn from chronic ingestion of alcohol. *Neurosci Lett* **309**:45-48.

Lyvers MF and Maltzman I (1991) Selective effects of alcohol on Wisconsin Card Sorting Test performance. *Br J Addict* **86**:399-407.

Majchrowicz E (1975) Induction of physical dependence upon ethanol and the associated behavioral changes in rats. *Psychopharmacologia* **43**:245-254.

Malfait AM, Gallily R, Sumariwalla PF, Malik AS, Andreakos E, Mechoulam R and Feldmann M (2000) The nonpsychoactive cannabis constituent cannabidiol is an oral anti-arthritic therapeutic in murine collagen-induced arthritis. *Proc Natl Acad Sci U S A* **97**:9561-9566.

Mansouri A, Demeilliers C, Amsellem S, Pessayre D and Fromenty B (2001) Acute ethanol administration oxidatively damages and depletes mitochondrial dna in

JPET Fast Forward. Published on May 5, 2005 as DOI: 10.1124/jpet.105.085779
This article has not been copyedited and formatted. The final version may differ from this version.

mouse liver, brain, heart, and skeletal muscles: protective effects of antioxidants. *J Pharmacol Exp Ther* **298**:737-743.

McDonough KH (2003) Antioxidant nutrients and alcohol. *Toxicology* **189**:89-97.

Nixon K and Crews FT (2002) Binge ethanol exposure decreases neurogenesis in adult rat hippocampus. *J Neurochem* **83**:1087-1093.

Obernier JA, Bouldin TW and Crews FT (2002a) Binge ethanol exposure in adult rats causes necrotic cell death. *Alcohol Clin Exp Res* **26**:547-557.

Obernier JA, White AM, Swartzwelder HS and Crews FT (2002b) Cognitive deficits and CNS damage after a 4-day binge ethanol exposure in rats. *Pharmacol Biochem Behav* **72**:521-532.

Paxinos G and Watson C (1986) *The rat brain in stereotaxic coordinates.* Academic Press Inc., San Diego.

Pfefferbaum A, Sullivan EV, Rosenbloom MJ, Mathalon DH and Lim KO (1998) A controlled study of cortical gray matter and ventricular changes in alcoholic men over a 5-year interval. *Arch Gen Psychiatry* **55**:905-912.

Pringle AK, Self J and Iannotti F (2000) Reducing conditions produce a loss of neuroprotective efficacy of competitive but not non-competitive antagonists in a model of NMDA-mediated excitotoxicity in organotypic hippocampal slice cultures. *Acta Neurochir Suppl* **76**:79-80.

Raghavendra V and Kulkarni SK (2001) Possible antioxidant mechanism in melatonin reversal of aging and chronic ethanol-induced amnesia in plus-maze and passive avoidance memory tasks. *Free Radic Biol Med* **30**:595-602.

Reddy SK, Husain K, Schlorff EC, Scott RB and Somani SM (1999) Dose response of ethanol ingestion on antioxidant defense system in rat brain subcellular fractions. *Neurotoxicology* **20**:977-987.

Schwartz PH and Wasterlain CG (1991) Cardiac arrest and resuscitation alters the pharmacokinetics of MK-801 in the rat. *Res Commun Chem Pathol Pharmacol* **73**:181-195.

Singh A, Naidu PS and Kulkarni SK (2003) Reversal of aging and chronic ethanol-induced cognitive dysfunction by quercetin a bioflavonoid. *Free Radic Res* **37**:1245-1252.

Sun AY and Sun GY (2001) Ethanol and oxidative mechanisms in the brain. *J Biomed Sci* **8**:37-43.

Switzer RC, 3rd (2000) Application of silver degeneration stains for neurotoxicity testing. *Toxicol Pathol* **28**:70-83.

Thirunavukkarasu V, Anuradha CV and Viswanathan P (2003) Protective effect of fenugreek (Trigonella foenum graecum) seeds in experimental ethanol toxicity. *Phytother Res* **17**:737-743.

Thomas JD, Fleming SL and Riley EP (2002) Administration of low doses of MK-801 during ethanol withdrawal in the developing rat pup attenuates alcohol's teratogenic effects. *Alcohol Clin Exp Res* **26**:1307-1313.

Wechsler H, Lee JE, Kuo M, Seibring M, Nelson TF and Lee H (2002) Trends in college binge drinking during a period of increased prevention efforts. Findings from 4 Harvard School of Public Health College Alcohol Study surveys: 1993-2001. *J Am Coll Health* **50**:203-217.

JPET Fast Forward. Published on May 5, 2005 as DOI: 10.1124/jpet.105.085779
This article has not been copyedited and formatted. The final version may differ from this version.

White AM (2003) What happened? Alcohol, memory blackouts, and the brain. *Alcohol Res Health* **27**:186-196.

Zou JY and Crews FT (2005) TNF alpha potentiates glutamate neurotoxicity by inhibiting glutamate uptake in organotypic brain slice cultures: neuroprotection by NF kappa B inhibition. *Brain Res* **1034**:11-24.

JPET Fast Forward. Published on May 5, 2005 as DOI: 10.1124/jpet.105.085779
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Footnotes

* These authors contributed equally to this work.

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Figure legends

Figure 1. Effect of Cannabidiol (CBD) on ethanol-induced neurotoxicity in rats. Gastric-cannulated rats were randomly divided into 6 groups: pair fed controls (Con, n=5), ethanol (ET, n=9), cannabidiol 20mg/kg (CBD20, n=6), cannabidiol 20mg/kg +ET (CBD-ET 20, n=8), CBD 40mg/kg (CBD40, n=6), and CBD 40mg/kg +ET (CBD-ET 40, n=8) and given ET 3 times daily in a binge ethanol model as per methods. CBD was given i.p. twice daily on days 2-4. 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 40mg/kg/day (bottom) treatment. Cells were stained by de Olmos silver staining method as per 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 in materials and methods. Data represent mean values ± SEM. An asterisk indicates a significant difference from control (Con), $p<0.05$, # indicates a significant difference from ethanol (ET), $p<0.05$, Mann Whitney U pair-wise comparisons. Data not shown for CBD treated controls, no degeneration was found. Scale bar = 100um.

Figure 2. Effect of NMDA receptor blockade on ethanol-induced neurotoxicity in rat hippocampus. Gastric-cannulated rats were randomly divided into 8 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 3 times daily in a binge ethanol model as per

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methods. Potential neuroprotectants were given s.c. in 6 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 ethanol (ET) administration and four hours following. A). Photomicrographs of hippocampus granule cell layer after 4 days of binge ethanol administration (ET, left) compared to control diet (Con, right) treated with either vehicle (top) or MK-801 0.6mg/kg/day (bottom). Cells were stained by De Olmos silver staining method as per methods section. B). Graphical representation of argyrophilic cell counting. Data represent mean values ± SEM. An asterisk indicates a significant difference from control (Con) $p<0.05$, # represents significant difference from ethanol (ET) $p < 0.05$, Mann Whitney U pair-wise comparisons. C). A second experiment was run as in figure 2B. Rats were divided into 4 groups: Con (n=6), ET (n=8), Lo MK (n=8), and Lo MK-ET (n=11) and given ET and MK-801 as above using 0.02mg/kg/day MK-801 in six divided doses. An asterisk indicates a significant difference from control (Con) $p<0.05$, # represents significant difference from ethanol (ET) $p < 0.05$, Mann Whitney U pair-wise comparisons. Scale bar = 100um.

Figure 3. Effect of NMDA receptor blockade on ethanol-induced neurotoxicity in rat entorhinal cortex. Experimental conditions as described in figure2. A). Photomicrographs of entorhinal cortex after 4 days of binge ethanol administration (ET, left) compared to control diet (Con, right) treated with either vehicle (top) or MK-801 0.6mg/kg/day (bottom). Cells were stained by De Olmos silver staining method as per methods section. B). Graphical representation of argyrophilic cell counting. 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), Mem + ET (Mem-ET, n=11). Data represent mean values ± SEM. An asterisk indicates a significant difference from control (Con) $p<0.05$, # represents significant difference from ethanol (ET) $p < 0.05$, Mann Whitney U pair-wise comparisons. C). A second experiment was run as in figure 2B, using 0.02mg/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 ± SEM. An asterisk indicates a significant difference from control (Con) $p<0.05$, # represents significant difference from ethanol (ET) $p < 0.05$, Mann Whitney U pair-wise comparisons. Scale bar = 200um.

Figure 4. A comparison of the antioxidative capacities of CBD, BHT and α-Tocopherol and their neuroprotective effects. A). The oxidation profiles of 1mM CBD, BHT, and α-Tocopherol when subjected to cyclic voltammetry. Data represent mean values ± SD from six replicates. Quantification of degeneration in rat hippocampus B) and Entorhinal Cortex C) following 4-day binge ethanol administration as described above in Fig 1. Rats were divided into 6 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 2-4. BHT was used at a dose of 40 mg/kg while TOC was administered at 80 mg/kg. Data represent mean values ± SEM. An asterisk indicates a significant difference from Con $p<0.05$, # indicates a significant difference from ET $p<0.05$, Mann Whitney U pair-wise comparisons. Data from BHT and TOC without ET treatment not shown, no degeneration seen.

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Figure 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 8 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 10mg / kg, bumetanide 1 mg / kg, and L-644,711 1 mg / kg. Data represent mean values ± SEM. An asterisk indicates a significant difference from Con $p<0.05$, # indicates a significant difference from ET $p<0.05$, Mann Whitney U pair-wise 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 Fig 5a, above. Data represent mean values ± SEM. An asterisk indicates a significant difference from Con $p<0.05$, # indicates a significant difference from ET $p<0.05$, Mann Whitney U pair-wise comparisons. Data from F, B and L without ET treatment not shown, no degeneration seen. C) The oxidation profile of 1mM furosemide, bumetanide and L-644,711 subjected to cyclic voltammetry. Data represent mean values ± SD from six replicates. D) The 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 10uM ferrous citrate catalyst). See Methods for experimental details. Data represent mean values ± SD from six replicates.

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Table 1. Blood alcohol levels (g/L).

Treatment group	Day 2	Day 3	Day 4	3 day avg
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

Rats were tail bled two hours after the first ethanol feeding of the day and blood alcohol levels were quantified using a standard alcohol dehydrogenase-based diagnostic kit. ET ethanol treated rats, CBD cannabidiol.

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Figure 1

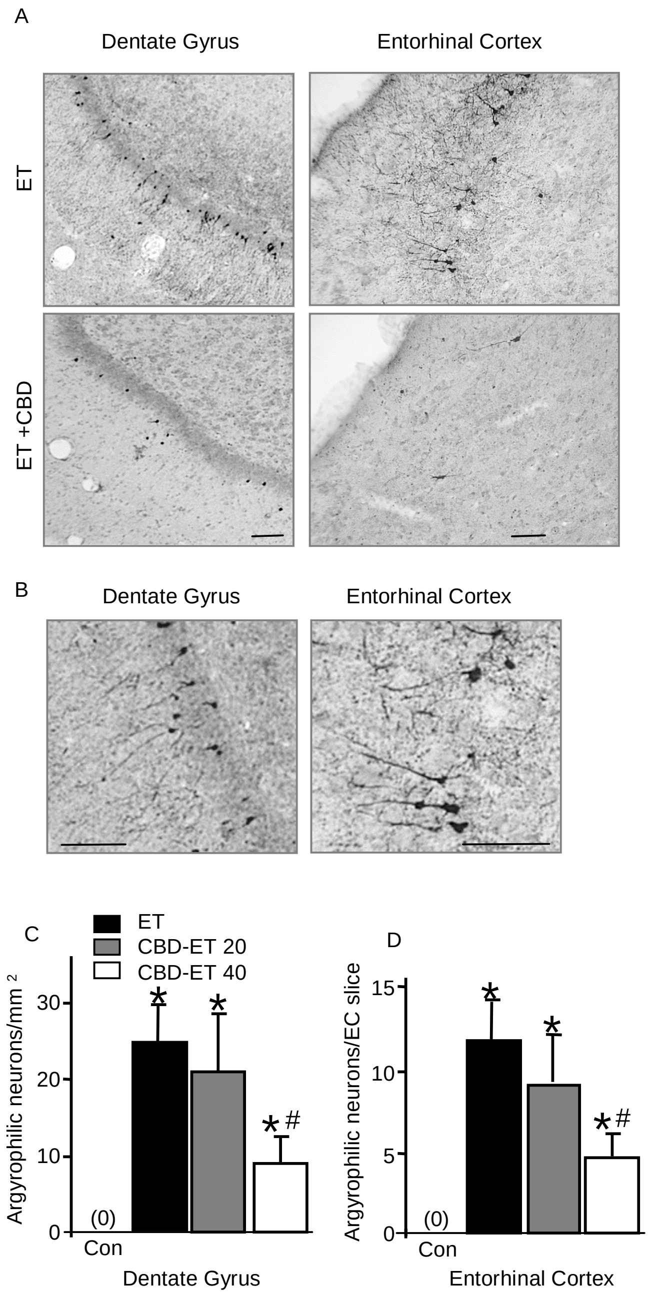

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Figure 2

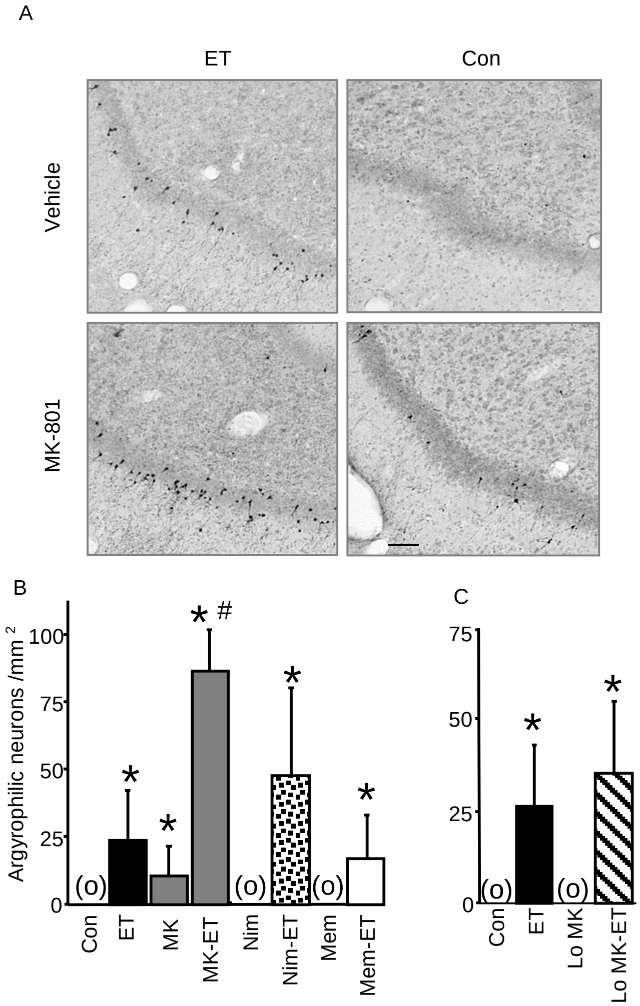

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Figure 3

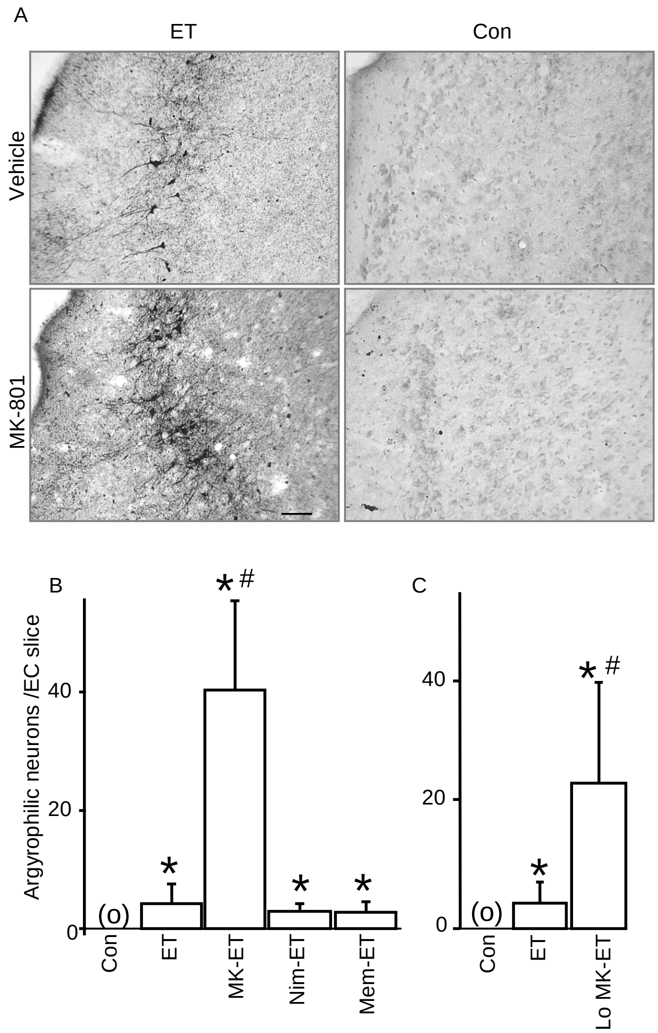

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Figure 4

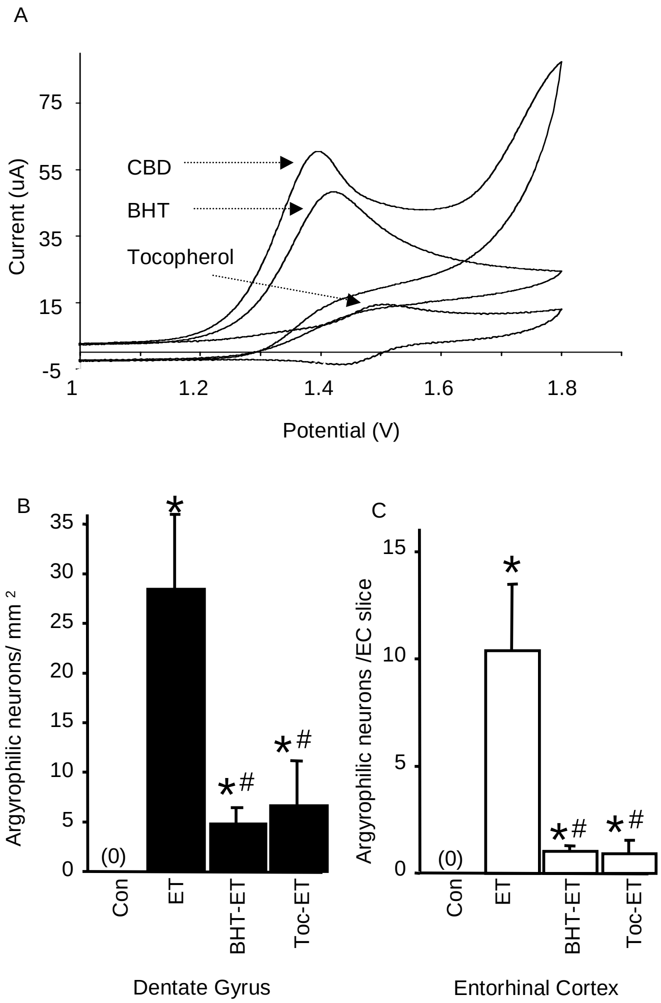

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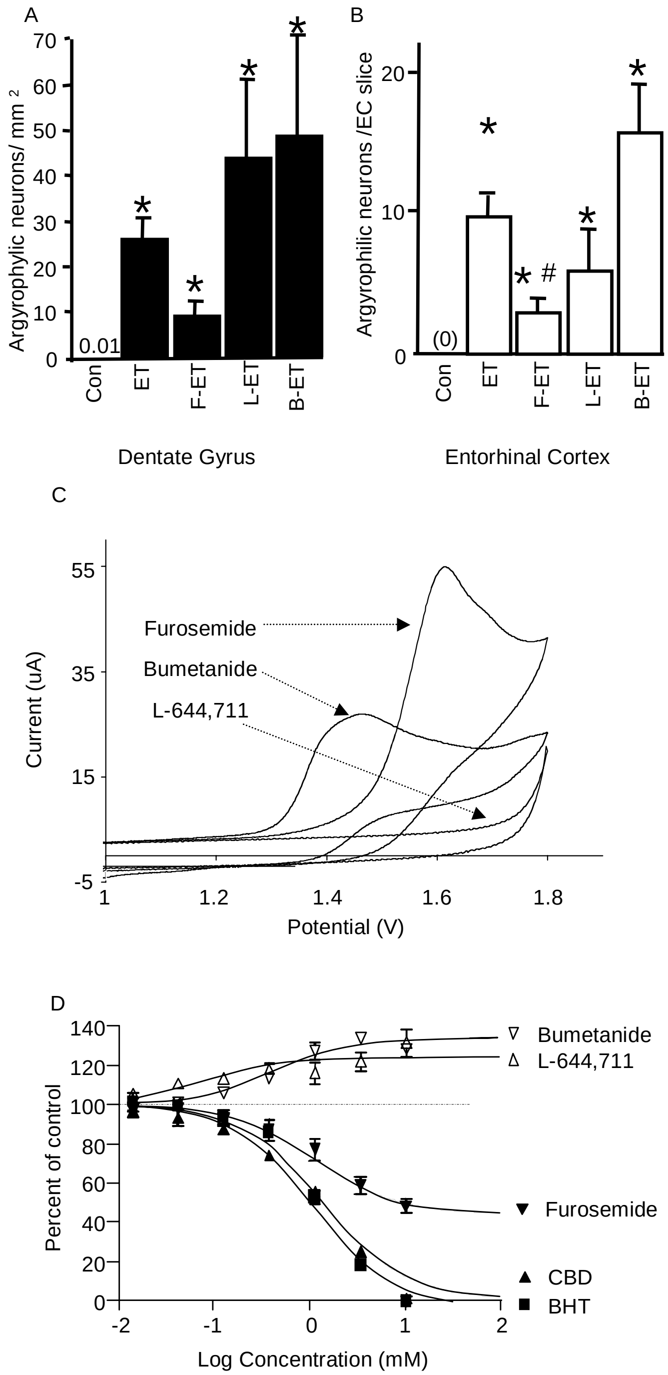
Figure 5



	BHT	CBD	Furosemide
EC50	1.1	1.4	1.1