Elevated Levels of Endocannabinoids and CB₁ Receptor-Mediated G-Protein Signaling in the Prefrontal Cortex of Alcoholic Suicide Victims

K. Yaragudri Vinod, Victoria Arango, Shan Xie, Suham A. Kassir, J. John Mann, Thomas B. Cooper, and Basalingappa L. Hungund

Background: Alcoholism is often comorbid with mood disorders and suicide. We recently reported an upregulation of CB₁ receptor-mediated G-protein signaling in the dorsolateral prefrontal cortex (DLPFC) of subjects with major depression who died by suicide. In the present study, we sought to determine whether the changes in depressed suicides would also be present in alcoholic suicides and whether the endocannabinoid (EC) system plays a role in suicide in alcoholism.

Methods: The density of CB₁ receptor and its mediated [³⁵⁵]GTPγS signaling were measured in the DLPFC of alcoholic suicides (AS) (n = 11) and chronic alcoholics (CA) (n = 11). The levels of ECs were measured by a liquid chromatograph/mass spectrometry.

Results: The CB₁ receptor density was higher in AS compared with the CA group in the DLPFC. Western blot analysis confirmed a greater immunoreactivity of the CB₁ receptor in AS. The CB₁ receptor-mediated [³⁵⁵]GTPγS binding indicated a greater signaling in AS. Higher levels of N-arachidonyl ethanolamide and 2-arachidonylethanolamide were observed in the DLPFC of AS.

Conclusions: The elevated levels of ECs, CB₁ receptors, and CB₁ receptor-mediated [³⁵⁵]GTPγS binding strongly suggest a hyperactivity of endocannabinoidergic signaling in AS. EC system may be a novel therapeutic target for the treatment of suicidal behavior.

Key Words: Alcoholism, suicide, human prefrontal cortex, endocannabinoids, CB₁ receptor, G-protein

Several natural lipids have emerged as candidate modulators of nervous system functions. For example, two putative endocannabinoids (ECs), N-arachidonyl ethanolamide (AEA) and 2-arachidonylethanolamide (2-AG), were shown to act as agonists for cannabinoid (CB) receptors (Devane et al 1992; Sugiura et al 1995), giving rise to the concept of an endocannabinoid system. The CB receptor family consists of two G-protein coupled receptors (GPCRs), CB₁ and CB₂. CB₁ receptors are localized primarily in neural tissue, whereas CB₂ receptors are located peripherally and are associated with the immune system. CB₁ receptors are known to inhibit adenyl cyclase (AC) (Howlett 1995; Childers and Breivogel 1998a) via Gi-protein and they are the most abundant neuromodulatory receptors in the brain, expressed in the cortex, hippocampus, cerebellum, and basal ganglia (Herkenham et al 1991; Glass et al 1997; Childers and Breivogel 1998b). High levels of ECs have also been found in the cortex, basal ganglia, and limbic structures, and this may suggest involvement of the EC system in cognitive, emotional, and motivational functions (Bisogno et al 1999).

Alterations in the monoaminergic neurotransmitter systems have long been implicated in the pathophysiology of depression and more recently in suicide (Arango et al 1990, 1995, 2002; Callado et al 1998; Gonzalez-Maeso et al 2002). Lower serotonergic activity may, to some degree, mediate genetic and developmental changes in suicide, aggression, mood, and alcoholism (for reviews, see Mann 1998, 2003). In recent years, the EC system has been slowly emerging as a target of psychiatric research, and alterations in CB₁ receptor levels in neurodegenerative diseases (Glass et al 1993; Westlake et al 1994) and schizophrenia (Dean et al 2001) have been reported. Recently, we reported elevated CB₁ receptor-mediated signaling in the dorsolateral prefrontal cortex (DLPFC) of depressed suicide victims (Hungund et al 2004). Our earlier animal studies have also suggested the participation of the EC system in alcoholism (for a review, see Hungund et al 2002).

Clinical evidence suggests that alcoholism and affective disorders are independently associated with suicide behavior (Roy 2000; Preuss et al 2002). Alcoholism and other substance use disorders are related to more aggression and impulsivity, also risk factors for suicide (Rosso and Amundson 1995; Rich et al 1998; Koller et al 2002). It has been suggested that chronic alcoholics are at 60 to 120 times higher risk for suicide than the general population, and alcoholism may contribute to about 25% of all suicides (Murphy and Wetzel 1990). Alcoholism and attempted suicide are familial, suggesting a role for genes and rearing (Potash et al 2000), although the responsible genes remain to be identified. An association between the CB₁ receptor gene and attention-deficit/hyperactivity disorder (ADHD) in alcoholics has also been recently reported (Ponce et al 2003). However, cellular mechanisms by which alcohol and the EC system produce behavioral changes in humans remain to be elucidated.

The modulation of the EC system by alcohol and the frequency of suicidal behavior in alcoholism raises the question whether the EC system plays a role in alcoholism or suicidal behavior per se. In this study, we have analyzed the density of CB₁ receptor, CB₁ receptor-mediated G-protein signaling and EC levels in the DLPFC, and CB₁ receptor-mediated G-protein signaling in the occipital cortex (OC) of alcoholic suicide victims and chronic alcohol control subjects dying from other causes.
Table 1. Characteristics of Alcoholic Suicide and Chronic Alcoholic Subjects

<table>
<thead>
<tr>
<th>Group</th>
<th>Sex (M/F)</th>
<th>Age (Years)</th>
<th>PMI (hours)</th>
<th>Cause of Death</th>
<th>Toxicology</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. CA</td>
<td>M</td>
<td>16</td>
<td>09</td>
<td>MVA, passenger</td>
<td>None</td>
</tr>
<tr>
<td>2. CA</td>
<td>M</td>
<td>17</td>
<td>12</td>
<td>MVA, passenger</td>
<td>None</td>
</tr>
<tr>
<td>3. CA</td>
<td>M</td>
<td>18</td>
<td>13</td>
<td>Homicide, stabbing</td>
<td>None</td>
</tr>
<tr>
<td>4. CA</td>
<td>M</td>
<td>24</td>
<td>09</td>
<td>Accident, drowning</td>
<td>CO</td>
</tr>
<tr>
<td>5. CA</td>
<td>F</td>
<td>30</td>
<td>13</td>
<td>Natural, cardiovascular</td>
<td>None</td>
</tr>
<tr>
<td>6. CA</td>
<td>F</td>
<td>35</td>
<td>13</td>
<td>Natural, cancer</td>
<td>None</td>
</tr>
<tr>
<td>7. CA</td>
<td>F</td>
<td>38</td>
<td>20</td>
<td>Homicide, GSW</td>
<td>None</td>
</tr>
<tr>
<td>8. CA</td>
<td>F</td>
<td>58</td>
<td>09</td>
<td>MVA, passenger</td>
<td>None</td>
</tr>
<tr>
<td>9. CA</td>
<td>M</td>
<td>64</td>
<td>12</td>
<td>Natural, cardiovascular</td>
<td>None</td>
</tr>
<tr>
<td>10. CA</td>
<td>M</td>
<td>67</td>
<td>09</td>
<td>Natural, hemorrhage</td>
<td>None</td>
</tr>
<tr>
<td>11. CA</td>
<td>M</td>
<td>70</td>
<td>08</td>
<td>Accident, fall from height</td>
<td>AE, BZ</td>
</tr>
</tbody>
</table>

Mean ± SD 39.7 ± 21.2 11.6 ± 3.4

The data are presented as mean and SD. No statistical significance was observed between age (t = .43, df = 20, p < .67) and PMI (t = .95, df = 20, p < .35) across the groups.

M, male; F, female; PMI, postmortem interval (rounded to nearest hours); CA, chronic alcoholic; MVA, motor vehicle accident; GSW, gunshot wound; CO, carbon monoxide; AE, antiepileptic; BZ, benzodiazapine; AS, alcoholic suicide.

Methods and Materials

Subjects, Tissue Collection, and Dissection

Brain samples of prefrontal cortex and occipital cortex were obtained from autopsy material in the Brain Collection of the Department of Neuroscience at the New York State Psychiatric Institute (NYSPI) and Columbia University, New York. All tissue used in this study was provided by the Allegheny County Coroner in accordance with protocols approved by the Institutional Review Board of the University of Pittsburgh, the New York State Psychiatric Institute, and Columbia University. Cortical tissues from Brodmann area 9 and visual association cortex (1.5 g each) were dissected frozen. The meninges and white matter were removed as much as possible, and tissues were returned immediately to −80°C until membrane preparation. Brain samples from 11 chronic alcoholics (CA) (age range: 16–70 years), who died from causes other than suicide, were studied together with a matched group of 11 subjects of alcoholic suicides (AS) who had a lifetime diagnosis of alcohol dependence or abuse. There were nine pairs of Caucasians and two pairs of African Americans. There were no significant differences in age, sex, and postmortem interval (PMI) distribution between AS and CA subjects. The demographic variables, such as sex, age, PMI, and cause of death, as well as toxicology, are summarized in Table 1.

Toxicological analyses were performed on all cases, ruling out recent consumption of substances of abuse (except alcohol [EtOH]) or psychoactive drugs (an antiepileptic and a benzodiazepine were detected in two cases [CA = 1 and AS = 1]). All cases were free of neuropathology. Both AS and CA subjects were characterized psychiatrically by interviews with family members and/or close friends using our previously validated psychological autopsy method and a structured clinical interview for Axis I and Axis II diagnoses according to DSM-III-R criteria (Kelly and Mann 1990). Psychological autopsies confirmed that all the subjects had a diagnosis of alcohol dependence or abuse. One of the suicides had Axis I diagnoses of major depression and schizoaffective disorder but was currently in remission. None of the subjects had comorbid nonalcohol substance use disorders. The samples were coded to mask investigators to group assignment.

Membrane Preparation

Brain tissue (~1 g) was homogenized in 20 volumes of ice-cold TME buffer (50 mM Tris-HCl, 3 mM magnesium chloride [MgCl2], and 1 mM of ethylenediaminetetraacetic acid [EDTA], pH 7.4) containing .32 M sucrose and a freshly added protease inhibitor cocktail. The homogenate was centrifuged at 1000g for 10 minutes at 4°C. The resulting supernatant was then centrifuged at 22,000g for 20 minutes. The pellet was dissolved in TME buffer and recentrifuged at 22,000g for 20 minutes. The final pellet was dissolved in TME buffer. Aliquots of membrane fractions were made and stored at −80°C until assay. The protein content of the membrane fraction was determined by Lowry et al (1951) method using bovine serum albumin (BSA) as the standard.

[^H]CP-55,940 Binding Assay

An aliquot of membrane (100 µg protein) was incubated with TME buffer,[^H]CP-55,940 (0.5–5.0 nM), and .1% fatty acid free BSA in silicone-treated test tubes for 1 hour at 37°C. The nonspecific binding of radioligand was defined by CP-55,940 (10 µM). The reaction was terminated by the addition of 2 mL ice-cold termination buffer (.1% BSA in 50 mM Tris-HCl, pH 7.4). The reaction mixture was rapidly filtered through polyethylenei-
CB₁ Receptor Immunoblot Analysis

Immunoblot analysis was used to confirm the presence of higher CB₁ receptor protein in the DLPFC of AS victims. An aliquot of membrane protein (30 μg) separated by 12% polyacrylamide gel was electrophoretically transferred to nitrocellulose membrane. The membrane was treated with blocking buffer (TTBS [10 mM Tris, .9% sodium chloride (NaCl); 1% Tween 20] containing 3% milk powder) of pH 7.4) for 1 hour at room temperature. The radioactivity was measured by liquid scintillation spectroscopy (Beckman Instruments, Fullerton, California) at an efficiency of 47% for tritium. The Bₐₘₐₓ (maximal binding sites) and Kᵦ (apparent dissociation constant) values were determined from saturation isotherms using nonlinear regression analysis to fit the data to the single binding equation.

Agonist-Stimulated [³⁵S]GTPγS Binding Assay

The CB₁ receptor-mediated [³⁵S]GTPγS binding assay was performed as described previously (Gonzalez-Maeso et al 2000) with minor modification. Briefly, an aliquot of membrane (50 μg protein) was preincubated in assay buffer (TME buffer and .1% fatty acid free BSA and 100 mM NaCl) containing guanosine 5′-diphosphate (GDP) (40 μM) in silicone-treated test tubes for 15 minutes. Later, [³⁵S]GTPγS (.05 nM) was added to the reaction mixture and incubated for 1 hour at 37°C. The CB₁ receptor agonist, CP-55,940 (1 μM), was used to study CB₁ receptor-mediated [³⁵S]GTPγS binding. A maximum stimulation of [³⁵S]GTPγS binding was achieved when cortical membranes were incubated with 1 μM CP-55,940 and 40 μM of GDP. The basal activity was estimated in absence of CP-55,940. To determine the specificity of CB₁ receptor-stimulated [³⁵S]GTPγS binding, a concentration-response effect in the presence of the CB₁ receptor antagonist SR141716A was also studied. The nonspecific binding of radioligand was determined in the presence of 10 μM GTPγS. The termination and filtration (without presoaking the filters in polyethyleneimine) of reaction mixture was performed as described for [³H]CP-55,940 binding assay. The radioactivity was measured by liquid scintillation spectroscopy at an efficiency of 95% for [³⁵S].

Measurement of EC Levels by Gas Chromatography/Mass Spectrometry

Endocannabinoid levels were measured by a liquid chromatography/mass spectrometry (LC-MS) method using the isotopic dilution procedure as described (Di Marzo et al 2000) with minor modifications. Briefly, brain tissue was homogenized in 4 mL of chloroform-methanol-Tris buffer (2:1:1, pH 7.4) containing .25 mM phenylmethylsulfonyl fluoride (PMSF), 20 μL of 1% butylated hydroxytoluene (BHT), 0.5 mL of AEA-d₈ (50 ng), and 0.50 mL of 2-AG-d₈ (500 ng). The homogenate was centrifuged and the organic layer removed and taken to dryness under nitrogen. The residue was dissolved in .3 mL of ethyl acetate and rechromatographed. The supernatant was transferred to another tube and dried down under nitrogen. The residue was redissolved in ethyl alcohol (40 μL) and transferred to a vial for the measurement of AEA and 2-AG by LC-MS (1100 series mass LC-MSD, Agilent Technologies, Wilmington, Delaware). The separation was achieved on a Supelcosil LC-8 column (Supelco, Bellefonte, Pennsylvania) (25 cm x 4.6 mm, 5 μm) using methanol-ammonium acetate-acetic acid (85:15:0.5) as a mobile phase. The standard curves were fitted with a quadratic equation with the curve encompassing a range of .5–50 and 50–1500 ng for AEA and 2-AG, respectively, and were processed similarly with quality controls with each batch of samples.

Figure 1. Scatchard transformation of saturation binding of [³H]CP-55,940 (.5–5.0 nm) to the prefrontal cortical membranes of a AS (alcoholic suicide) and CA (chronic alcoholic) subjects. The inset represents the Scatchard transformation of the same binding data.

Figure 2. The density of CB₁ receptor was estimated using [³H]CP-55,940 in the prefrontal cortical membranes of AS (alcoholic suicide, n = 11) and CA (chronic alcoholic, n = 11) subjects. **Significantly higher than that of CA subjects (t = 3.59, df = 18, p < .001).
Also revealed a significantly higher (67%, density may not be due to its altered affinity. Western blot analysis
significant difference in the affinity of receptor between the groups
/H11006
fmol/mg protein) compared with CA (590.2
/H11005
protein) (Figure 3B). Furthermore, significant positive correlations between
activity) of the CB1 receptor were observed in both CA (34%;
Figure 3B). The CB1 receptor-mediated 
/H11006
S binding was observed in cortical membranes of AS (102.1 ± 10.5 fmol/mg protein) compared with CA (76.4 ± 11.8
/H11005
protein) subjects (Figure 4). However, this parameter
upregulation of receptor
expression over the basal. ***Significantly higher than that of CA
subjects (t = 2.66, df = 20, p < .01).

Statistical Analyses
Statistical analysis was performed by using GraphPad Prism (GraphPad software, San Diego, California). Experiment in each sample was run in at least duplicate unless otherwise indicated. All values presented are mean and standard deviation. Immuno-
blots were analyzed using the National Institutes of Health (NIH) image software program. The demographic variables (age and
/PMI) were compared between CA and AS subjects with independent t tests. Differences in binding parameters, protein, and EC levels between CA and AS groups as a whole were analyzed using independent-sample unpaired t test with two-tailed p values considered significant at p < .05.

Results

The Density of CB1 Receptor: Radioligand Binding and
Immunoblot Analyses
The saturation binding analysis suggests that [3H]CP-55,940
binding sites were saturable at 5.0 nM concentration (Figure 1). The nonspecific binding was 10% to 18% of total [3H]CP-55,940 binding. A Scatchard analysis demonstrates high affinity (apparent dissociation constant [Kd] was 1.3 ± .3 nm), saturable, monophasic binding, and a Hill coefficient of near unity indicates a single class of receptor at the concentrations used. Significantly higher (39%) density (Bmax) of the CB1 receptor was observed in the DLPFC of AS (820.1
/H11005
42.5 pmol/gm tissue) were significantly lower than 2-AG (2.20
/H11006
pmol/gm tissue) (t = 2.66, df = 20, p = .014) groups. A representative CB1 receptor immunoblot of a CA and matched AS is provided in Figure 3A. The Western blot analysis clearly demonstrated single immunoreactivity band, which corresponds to the relative molecular weight of the CB1 receptor.

CB1 Receptor-Mediated [35S]GTPγS Binding

CB1 receptor agonist, CP-55,940-stimulated [35S]GTPγS binding assay was performed in membranes isolated from the DLPFC and the OC of CA and AS subjects to assess the functional coupling between CB1 receptor and G-protein. Greater stimulation (34%; t = 5.38, df = 20, p = .0001) of CB1 receptor-mediated
/H9253
S binding was observed in cortical membranes of AS (102.1 ± 10.5 fmol/mg protein) compared with CA (76.4 ± 11.8
/H11005
protein) subjects (Figure 4). However, this parameter
upregulation of receptor
expression over the basal. ***Significantly higher than that of CA
subjects (t = 2.66, df = 20, p < .01).

Levels of Endocannabinoids
Levels of both ECs, AEA and 2-AG, were measured in the DLPFC of AS and CA subjects. The levels of AEA (98.4 ± 26.4
/H11005
pmol/gm tissue) were significantly lower than 2-AG (2.20 ± .80
/H11006
nmol/gm tissue) in the DLPFC of CA subjects. Levels of both AEA (79%, 176.8 ± 42.5 pmol/gm tissue, t = 5.18, df = 20, p = .0001) and 2-AG (47%, 3.24 ± .94 nmol/gm tissue, t = 2.75, df = 20, p
The observed upregulation of CB1 receptors may reflect in the DLPFC of AS, corroborating the results of radioligand binding analysis demonstrated a higher immunoreactivity for CB1 receptor membranes of AS as compared with CA. In addition, immunoblot revealed a 1.3-fold higher level of CB1 receptor protein in AS compared with CA. The consequence of elevated levels of CB1 receptors in the DLPFC of AS observed in this study is not known. Upregulation of CB1 receptors due to the feedback or a homeostatic response to low levels of ECs is not the explanation, because we observed elevated levels of both AEA and 2-AG in the DLPFC of AS. Elevated levels of both ECs (Leweke et al 1999; De Marchi et al 2003) and CB1 receptors have also been found in schizophrenic patients (Dean et al 2001), indicating that this pattern of abnormality may not be unique to suicide. However, it is not clear whether the alterations reported in these studies were associated with suicidal behavior or with schizophrenia. Delta-9-tetrahydrocannabinol, a primary constituent of marijuana, which is a CB1 receptor agonist and mimics the action of ECs, appears to increase certain forms of impulsive behaviors in humans (McDonald et al 2003). The high levels of ECs/CB1 receptor may facilitate impulsive behavior, which is characteristic of chronic alcoholics and depressed subjects with suicidal behavior. Impulsivity is thought to be part of a diathesis for suicidal and aggressive behaviors, increasing the probability of acting on such feelings (for a review, see Mann et al 1999).

The regulatory mechanisms governing the EC system remain largely unknown. Neuroanatomical and electrophysiological studies of the mammalian nervous system have revealed that the CB1 receptors are located in presynaptic terminals of neurons (Kreitzer and Regehr 2001). Fatty acid amide hydrolase (FAAH), a membrane bound serine hydrolase that inactivates ECs, appears to be preferentially located in the somatodendritic compartments of neurons. Endocannabinoids are released on demand by stimulated neurons and activate presynaptic CB1 receptors. Based on these findings, it has been suggested that ECs are synthesized by postsynaptic cells that act as retrograde messengers to modulate neurotransmitter release (Kreitzer and Regehr 2001; Wilson and Nicoll 2002). Therefore, there may be elevated retrograde signaling mediated by the EC system in the DLPFC of suicide victims.

The relevance of impaired cannabinoidergic signaling to the pathophysiology of suicide remains to be elucidated. The
sensitization of cannabinoidergic signaling could lead to decreased cyclic adenosine monophosphate (cAMP) content of the cell, as these receptors are negatively coupled to AC, resulting in the reduced cAMP dependent protein kinase A (PKA) and cAMP response element binding protein (CREB) activities. A reduced activity of cAMP/PKA and CREB signaling in the cortex of suicide brain has been reported (Dowlatshahi et al 1999; Reiaich et al 1999; Dwivedi et al 2002a, 2003). Many biological responses are regulated by the state of phosphorylation of specific proteins, which in turn affects the regulation of cellular functions. Thus, a hyperactivity of cannabinoidergic signaling may result in impairments in various physiological functions that manifest in suicidal behavior.

Several studies have highlighted the effect of age, sex, and PMI on the levels and function of receptors and G-proteins (Dowlatshahi et al 1999) and also the effect of PMI on the levels EGCs (Schmid et al 1995). However, the brain samples analyzed in this study were closely matched with regard to sex, age, ethnic background, and PMI, and no significant group differences in these demographic variables were observed. Suicide is associated with several psychiatric disorders and also drugs of abuse (Suominen et al 1996; Rich et al 1998; Kessler et al 1999; Garlow 2002). However, all the subjects included in this study had a diagnosis of alcoholism and no drug of abuse other than ETOH was detected postmortem, and yet the elevation in CB1 receptors occurred only in the suicide group. Whether the observed alterations in the DLPFC of AS is due to the pathophysiology of suicide or psychotropic medication is of particular relevance. However, our toxicological analyses revealed that only two cases (one from each group) had medications (antiepileptic and benzodiazapine) at the time of death and no other psychoactive drugs were detected in remaining subjects. Therefore, the present findings in brains of AS are unlikely to be the result of short-term drug effects and more likely to be related to the pathophysiology of suicide. In the present study, we included CA as a psychiatric control group for AS instead of normal control groups. This enabled us to identify the biochemical alterations in the brain of alcoholic suicide victims with greater specificity. Alteration of CB1 receptor-mediated G-protein signaling seems to be region-specific because the CB1 receptor-mediated [35S]GTPγS signaling was found to be unaltered in the OC of AS victims. This may be due to unaltered density of CB1 receptor in the OC of AS victims. Future studies are needed to examine the components of EC system in the human postmortem brains of alcoholics, alcoholic suicides, and normal control subjects in more brain regions of interest to further understanding the role of EC system in the neurobiology of alcoholism and suicide.

In conclusion, convergence of three different approaches pointing to the upregulation of CB1 receptors taken together with higher levels of EGCs strongly suggests that hyperactivity of the EC signaling in the DLPFC may be a potential factor in suicide in alcoholism. The present study corroborates our previous observations and further elucidates the association between hyperactivity of EC system and suicide. Pharmacological agents that modulate EC function would be of interest in the potential development of treatment for suicidal behavior.

This study was supported by Grants AA13003 and National Alliance for Research on Schizophrenia and Depression, Independent Investigator Award (BLH); AA09004 and MH40210 (VA); MH62185 (JJM); and American Foundation for Suicide Prevention Young Investigator Grant (KYY).

We thank Dr. Veeramma, Center for Dementia Research, Nathan Kline Institute, for his technical advice.


