SPECIAL ISSUE ARTICLE
ROLE OF THE ENDOCANNABINOID SYSTEM IN THE DEVELOPMENT OF
TOLERANCE TO ALCOHOL
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Abstract — The present review evaluates the evidence that the endocannabinoid system plays in the development of tolerance to alcohol. The identification of a G-protein-coupled receptor, namely, the cannabinoid receptor (CB1 receptor), which was activated by Δ9-tetrahydrocannabinol (Δ9-THC), the major psychoactive component of marijuana, led to the discovery of endogenous cannabinoid agonists. Until now, four fatty acid derivatives identified to be arachidonylethanolamide (AEA), 2-arachidonylglycerol (2-AG), 2-arachidonoylglycerol ether (noladin ether) and virodhamine have been isolated from both nervous and peripheral tissues. Both AEA and 2-AG have been shown to mimic the pharmacological and behavioural effects of Δ9-THC. The role of the endocannabinoid system in the development of tolerance to alcohol was not known until recently. Recent studies from our laboratory have implicated for the first time a role for the endocannabinoid system in development of tolerance to alcohol. Chronic alcohol treatment has shown to down-regulate CB1 receptors and its signal transduction. The observed downregulation of CB1 receptor function results from the persistent stimulation of the receptors by AEA and 2-AG, the synthesis of which has been shown to be increased by chronic alcohol treatment. The enhanced formation of endocannabinoids may subsequently influence the release of neurotransmitters. It was found that the DBA/2 mice, known to avoid alcohol intake, have significantly reduced CB1 receptor function in the brain, consistent with other studies in which the CB1 receptor antagonist SR141716A has been shown to block voluntary alcohol intake in rodents. Similarly, activation of the CB1 receptor system promoted alcohol craving, suggesting a role for the CB1 receptor gene in excessive alcohol drinking behaviour and development of alcoholism. Ongoing investigations may lead to a better understanding of the mechanisms underlying the development of tolerance to alcohol and to develop therapeutic strategies to treat alcoholism.

INTRODUCTION
Alcohol dependence is a leading cause of morbidity and various medical and socio-economic problems. It is defined by compulsive, excessive use of alcohol despite negative consequences. Alcohol dependence is usually accompanied by tolerance to the intoxicating effects of alcohol and by withdrawal symptoms including tremors and confusion when consumption of alcohol ceases. Although important advances have been made in recent years in understanding the mechanisms underlying the development of tolerance to and dependence on alcohol, the exact mechanisms are still elusive. The present article reviews the role played by the endocannabinoid system in the molecular mechanism involved in the development of alcohol tolerance, which possibly influences alcohol-drinking behaviour.

The endocannabinoid system comprises cannabinoid receptors, endogenous cannabinoids and the molecules involved in the inactivation of endocannabinoids (uptake and degradation enzyme known as fatty acid amidase hydrolase, FAAH). Cannabinoid receptors belong to the large family of seven transmembrane-spanning (7TM) G-protein-coupled receptors (GPCRs). As a class, GPCRs are of fundamental physiological importance, mediating the actions of most known neurotransmitters and hormones. Cannabinoid receptors are intriguing members of this receptor family. There are two types of cannabinoid receptors, CB1 and CB2, defined by their unique localization. The CB1 receptor is widely distributed in several regions of the brain (Herkenham et al., 1990), with a high density in the cortex, hippocampus, basol ganglia and cerebellum. Both CB1 and CB2 receptors have been characterized and cloned (Howlett et al., 2002). The functional response of the CB1 and CB2 receptors is coupled via Gi/Go proteins, negatively to adenylate cyclase and N- and P/Q-type Ca2+ channels. They are positively coupled to A-type and inwardly rectifying K+ channels and mitogen-activated protein kinases (Basavarajappa and Hungund, 2002; Howlett et al., 2002).

In 1992, Devane et al. showed the existence of an endogenous cannabimimetic substance in the mammalian brain, found to bind the CB1 receptor; and it was characterized to be arachidonylethanolamide (anandamide, AEA). Since then, three other endocannabinoids such as 2-arachidonylglycerol (2-AG) (Devane et al., 1992; Mechoulam et al., 1995; Sugiuara et al., 1995), 2-arachidonoylglycerol ether (noladin ether) and virodhamine (Porter et al., 2002) have been identified.

Unlike classical neurotransmitters and neuropeptides, AEA and 2-AG are not stored in intracellular compartments but are produced on demand by receptor-stimulated cleavage of lipid precursors (Di Marzo et al., 1994; Cadas et al., 1997; Mechoulam et al., 1998; Basavarajappa and Hungund, 1999a; Basavarajappa et al., 2000, 2003) and released from neurons immediately afterwards (Di Marzo et al., 1994; Mechoulam et al., 1998; Basavarajappa and Hungund, 1999a; Giuffrida et al., 1999; Basavarajappa et al., 2000, 2003). The AEA precursor is an N-arachidonoylphosphatidylethanolamine (N-APe), which is believed to originate from the transfer of arachidonic acid (AA) from the sn-1 position of 1,2-sn-di-arachidonoylphosphatidylcholine to phosphatidylethanolamine,
catalysed by a calcium-dependent transacylase (CDTA). N-ArPE is then cleaved by an N-acylphosphatidylethanolamine (NAPE)-specific phospholipase D (PLD) (Natarajan et al., 1981; Schmid et al., 1983; Di Marzo et al., 1994), which releases AEA and phosphatidic acid. The biosynthesis of 2-AG has been shown to occur by two possible routes in neurons. Phospholipase C (PLC)-mediated hydrolysis of membrane phospholipids produces diacylglycerol (DAG), which may be converted subsequently to 2-AG by diacylglycerol lipase (DGL) activity. Alternatively, phospholipase A1 (PLA1) may generate a lysophospholipid, which may be hydrolyzed to 2-AG by lyso-PLC activity. AEA and 2-AG are inactivated by the reuptake by a membrane transport molecule, the AEA membrane transporter (AMT) (Beltramo et al., 1997; Hillard et al., 1997; Maccarrone et al., 1998; Beltramo and Piomelli, 2000; Hillard and Jarrahian, 2000; Giuffrida et al., 2001; Basavarajappa et al., 2003) and by intracellular enzymatic degradation (Di Marzo et al., 1994; Day et al., 2001; Deutsch et al., 2001) through FAAH-mediated hydrolysis (Cravatt et al., 1996; Beltramo and Piomelli, 2000; Ueda et al., 2000; Bisogno et al., 2001; Deutsch et al., 2001; Fowler et al., 2001). The metabolism, pharmacology and physiology of AEA and 2-AG has been covered elsewhere in this issue in detail (Rodríguez de Fonseca et al., 2004).

**Alcohol and endocannabinoids**

In the brain, the presence of the endocannabinoid signalling system in the thalamus, hippocampus and cortex or in the striatum, substantia nigra and cerebellum supports a role for the endogenous cannabinoid-signalling system in cognitive and motor responses. The anatomical distribution and actions of endocannabinoids is consistent with the behavioural effects of alcohol, including memory disruption, decrease in motor activity, catalepsy, antinociception and hypothermia (Ryan and Butters, 1980; Brandt et al., 1983; Gebhardt et al., 1984; Herkenham et al., 1991b; Compton et al., 1993; Fadda and Rossetti, 1998). Adaptation in several steps of the endocannabinoid system in the brain may play an important role in the development of tolerance to and dependence on alcohol (Basavarajappa et al., 1998a; Basavarajappa and Hungund, 1999a,b; Hungund and Basavarajappa, 2000a,c).

In the last seven years, several studies, including those from our laboratory, provided evidence for the participation of the endocannabinoid system in the pharmacological actions of alcohol and in alcohol-drinking behaviour. In our earlier studies, we demonstrated that chronic alcohol exposure leads to the activation of Ca2+-dependent and the arachidonic acid-specific phospholipase A2 (PLA2), a key enzyme involved in the formation of endocannabinoids in neuronal cells and the brain (Basavarajappa et al., 1997, 1998b). Later, we extended these studies to examine the chronic effect of alcohol on the endocannabinoids in an *in vitro* system. Indeed, it was found that the exposure of SK-N-SH cells or cerebellar granular neurons (CGNs) to chronic alcohol resulted in the increased accumulation of AEA (Basavarajappa and Hungund, 1999a; Basavarajappa et al., 2003) and 2-AG (Table 1; Basavarajappa et al., 2000). In these studies, we demonstrated that the synthesis of AEA and 2-AG increased with increasing duration of alcohol exposure, peaking at 72 h with 100 mM alcohol, the experimental condition known to cause cellular tolerance and dependence to alcohol in neurons. These adaptive changes were further increased by the Ca2+-ionophore or ionomycin and inhibited by pertussis toxin (which selectively inactivates G-protein) and the CB1 receptor antagonist SR 141716A, which is also shown to inhibit alcohol drinking in rodents (Arnone et al., 1997; Colombo et al., 1998; Gallate and McGregor, 1999; Rodríguez de Fonseca et al., 1999a; Freedland et al., 2001). In a related study, Swiss–Webster male mice were made alcohol tolerant by inhalation of alcohol vapours for 72 h (Goldstein, 1972) and the lipids were extracted from the brains of the decapitated mice. The AEA fraction was purified chromatographically. Characterization and quantification were achieved by the gas chromatographic-mass spectral (GC-MS) method using the chemical ionization-single ion monitoring technique (CI-SIM). These results showed that chronic alcohol exposure led to a significant increase in the levels of AEA in the brain and a significant decrease in N-ArPE, an immediate precursor to 2-AG, which is also shown to inhibit alcohol drinking in rodents (Arnone et al., 1997; Colombo et al., 1998; Gallate and McGregor, 1999; Rodríguez de Fonseca et al., 1999a; Freedland et al., 2001). In a related study, Swiss–Webster male mice were made alcohol tolerant by inhalation of alcohol vapours for 72 h (Goldstein, 1972) and the lipids were extracted from the brains of the decapitated mice. The AEA fraction was purified chromatographically. Characterization and quantification were achieved by the gas chromatographic-mass spectral (GC-MS) method using the chemical ionization-single ion monitoring technique (CI-SIM). These results showed that chronic alcohol exposure led to a significant increase in the levels of AEA in the brain and a significant decrease in N-ArPE, an immediate precursor to AEA synthesis, compared with the levels in control brains (Hungund et al., 2002). A recent study also demonstrated that chronic alcohol exposure in rats caused a decrease in the content of both AEA and 2-AG in the midbrain, whereas AEA content increased in the limbic forebrain, a key area for the reinforcing properties of habit-forming drugs, including alcohol (Gonzalez et al., 2002b). Although the levels of endocannabinoids are lower in normal tissues, their levels were found to increase significantly during movement disorders, cell injury and tissue degeneration, and during the postmortem period (Schmid et al., 1995; Felder et al., 1996; Kempe et al., 1996). Selective increase in the formation of AEA in the limbic forebrain has also been observed in Δ9-THC-tolerant rats (Di Marzo et al., 2000b) and in mouse neuroblastoma cells treated with Δ9-THC (Hunter and Burstein, 1997). These observations point to the possible involvement of the endocannabinoids in the alcohol-induced neuroadaptive changes in these cells. These observations suggest the possible involvement of the endocannabinoids in the alcohol-induced neuroadaptive changes in the brain, and that change in endocannabinoid-mediated neurotransmission

<table>
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<tr>
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<th>Control</th>
<th>Alcohol</th>
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<tbody>
<tr>
<td>AEA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal (µM)</td>
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<td>100.0 ± 5.8</td>
</tr>
<tr>
<td>SR 141716A (µM)</td>
<td>91.9 ± 10.9</td>
<td>91.9 ± 10.9</td>
</tr>
<tr>
<td>PTX (100 ng/ml)</td>
<td>106.8 ± 8.4</td>
<td>106.8 ± 8.4</td>
</tr>
<tr>
<td>2-AG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal (µM)</td>
<td>100.0 ± 5.8</td>
<td>100.0 ± 5.8</td>
</tr>
<tr>
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<td>106.8 ± 8.4</td>
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Table 1. Chronic alcohol enhances endocannabinoid levels in neuronal cells
may be responsible for the activation of the reward system by alcohol.

The mechanism by which chronic alcohol exposure leads to a selective increase in the levels of AEA and 2-AG remains to be established. AEA is an ethanalamide derivative of AA. Arachidonic acid is derived from membrane lipids by selective activation of Ca²⁺-dependent PLA₂, which releases the Sn-2-arachidonyl moiety from the membrane PL₄ phosphatidylcholine (PC) (Basavarajappa et al., 1997, 1999b). In our studies, the formation of N-ArPE was accompanied by concurrent formation of AEA, suggesting that the N-ArPE-specific phospholipase D (PLD) may be constitutively active in chronic alcohol-exposed SK-N-SH cells. Although several studies have shown the activation of PLD in various alcohol models (Kiss, 1992; Lundqvist et al., 1994; Gustavsson, 1995), the involvement of an N-ArPE specific PLD-like enzyme remains to be established. It is also possible that the increased accumulation of AEA is the result of inhibition of FAAH or transport of AEA by chronic alcohol. The exact mechanism by which 2-AG is synthesized has not been clearly established. As discussed elsewhere in this issue, three main biochemical pathways exist in the neurons for the formation of 2-AG through the sequential action of a PI-specific phospholipase C, sn-1-diacylglycerol lipase and phospholipase C (Bisogno et al., 1997, 1999; Stella et al., 1997; Di Marzo et al., 1998). The FAAH enzyme inhibitor phenylmethylsulfonylfluoride (PMSF) was shown to inhibit the hydrolysis of 2-AG in neurons and was effective in increasing the accumulation of 2-AG in both the control and alcohol-exposed CGNs (Basavarajappa et al., 2000). In this study, the 2-AG accumulation was not affected by the more selective FAAH inhibitor E-6- (bromomethylene) tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one (BTNP), which is in agreement with the previous observation that BTNP (5 μM) had no effect on 2-AG accumulation or metabolism in human astrocytoma cells (Beltramo and Piomelli, 2000). Therefore, the possibility remains for the existence of a specific lipase for 2-AG hydrolysis, which may be responsible for the physiological inactivation of 2-AG in neurons (Sugiuira et al., 2002). In our recent study, glutamate, which is also known to stimulate the formation of N-acylthanolamides (NAEs) in cortical neurons (Hansen et al., 1995), stimulated the formation of 2-AG in CGNs (Basavarajappa et al., 2000). However, glutamate did not cause further enhancement of the alcohol-induced formation of 2-AG, suggesting that alcohol might have caused a saturation of 2-AG levels, which could not be increased further by glutamate. However, the alcohol-induced formation of 2-AG could be inhibited by the N-methyl-D-aspartate (NMDA) receptor antagonist MK-801. Interestingly, the CGNs exhibited a 100% increase in [Ca²⁺], after 4 days of exposure to alcohol (Iorio et al., 1992), suggesting that the glutamate-induced increase in intracellular Ca²⁺ may be responsible for the formation of 2-AG by CGNs. This also suggests that chronic alcohol-induced activation of the NMDA receptor may trigger an increase in cytoplasmic Ca²⁺ concentration, which in turn may be responsible for the enhanced synthesis of 2-AG. The dopamine (D2) receptor agonist 7-H-DPAT did not enhance the formation of 2-AG either in control or in alcohol-exposed CGNs, whereas co-treatment of CGNs with the D2 receptor antagonist haloperidol inhibited the alcohol-induced formation of 2-AG, which suggests the interaction of the D2 receptor system or the direct action of this compound with the alcohol-induced formation of 2-AG. The D2 receptor activation by the agonist quinpirole led to an 8-fold stimulation of AEA release in the striatum without affecting the 2-AG release, and this stimulation was inhibited by the antagonist raclopride (Giufrida et al., 1999). In another study, the administration of quinpirole to reserpine-treated rats reduced 2-AG and AEA levels in the globus pallidus but did not alter them significantly in any other region of the brain (Di Marzo et al., 2000b). These results suggest the possible differential regulation of endocannabinoids, which may influence the formation of different endocannabinoids in different regions of the brain. Further studies to elucidate the mechanisms leading to the synthesis and degradation of AEA and 2-AG will be of great use in the treatment of problems associated with drugs of abuse, including alcohol.

AEA signalling at the cannabinoid CB₁ receptors is terminated by an uptake mechanism that transports AEA into the cell, where it subsequently undergoes rapid degradation by FAAH (Cravatt et al., 1996; Beltramo et al., 1997; Hillard et al., 1997; Piomelli et al., 1999). Based on the available data, it is suggested that AEA uptake is a carrier-mediated process that is time- and temperature-dependent and saturable, and is inhibited by unique pharmacologic agents (Di Marzo et al., 1994; Beltramo et al., 1997; Hillard et al., 1997; Hillard and Jarrahian, 2000; Rakshan et al., 2000). Co-localization of both FAAH and CB₁ receptors in the brain may point to a possible role of FAAH in AEA signalling and uptake (Egertova et al., 1998). Thus, chronic alcohol-induced increases in extracellular AEA could result in a decrease in AEA influx, an increase in AEA efflux from the cell, and/or altered intracellular metabolism (Basavarajappa et al., 2003). In our recent study, we investigated the chronic and acute effects of alcohol on AEA transport in CGNs (Basavarajappa et al., 2003). We found that chronic exposure to alcohol leads to an increase in extracellular AEA by inhibiting the uptake of AEA. This effect was independent of the CB₁ receptor since CB₁ receptor knockout mice have normal uptake activity (Basavarajappa et al., 2003). After prolonged exposure to alcohol, cells become tolerant to the effects such that AEA uptake is no longer inhibited by acute alcohol (Fig. 1; Basavarajappa et al., 2003). Chronic exposure to alcohol did not show any direct inhibition of FAAH activity in these neurons. These data suggest that alcohol-induced inhibition of AEA uptake may, in part, be responsible for the alcohol-induced increase in extracellular AEA.

**Alcohol and cannabinoid receptors**

Further, we found that chronic exposure to alcohol not only increases the endocannabinoids in neuronal cells and brain but also impairs the CB₁ receptor function in the brain (Basavarajappa et al., 1998a; Basavarajappa and Hungund, 1999b). The results of this study indicate that the chronic alcohol exposure decreased the Bₘₐₓ of CB₁ receptors and inhibited the ability of the CB₁ receptor agonist to stimulate GTPγS binding in mice (Table 2), possibly through sustained higher levels of endocannabinoids (Basavarajappa and Hungund, 1999a; Basavarajappa et al., 2000; Hungund et al., 2002). The observed downregulation of the CB₁ receptor signalling system by chronic alcohol may also result from
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over-stimulation of receptors through increased synthesis of the endogenous CB1 receptor agonist (AEA and or 2-AG). These observations are consistent with the recent data, which indicate that forced consumption of high levels of alcohol significantly decreases CB1 receptor gene expression in the brains of mice treated with chronic alcohol (Wand et al., 1993). These results strongly support the participation of the endocannabinoid system in mediating some of the pharmacological and behavioural effects of alcohol, and the CB1 receptor may thus constitute an important target for therapeutic intervention in alcohol-related behaviours.

As discussed previously, the primary actions of cannabinoids are mediated through G-protein-coupled receptors and an intracellular signalling mechanism that initiates cellular response by cannabinoid-activated G-proteins (Matsuda et al., 1990; Howlett, 1995; Howlett et al., 2002). Cannabinoid inhibition of adenylate cyclase plays an important role in several aspects of cannabinoid functions, such as modulation of conductance at a voltage-dependent K+ channel (Caulfield and Brown, 1992; Mackie and Hille, 1992), thus providing an effective rationale for the behavioural effects of cannabinoids (Howlett et al., 1986). Further studies to examine whether the chronic alcohol-mediated downregulation of brain CB1 receptors has any functional effect on CB1 receptor-activated G-proteins revealed that the net CB1 receptor agonist (CP-55,940)-stimulated [35S]GTPγS binding was reduced significantly in chronic alcohol-exposed mice without any significant changes in the G-protein affinity using alcohol vapour inhalation. In both the models, animals have been shown to exhibit tolerance to alcohol (Goldstein and Pal, 1971; Goldstein, 1980; Goldstein and Zaechele, 1983; Gate and Lal, 1999; Rasmussen et al., 2002). Various agonists acting at various receptors coupled through G proteins to adenylate cyclase have been shown to be reduced by alcohol (Rabin, 1990).

Table 2. Alcohol tolerance downregulates CB1 receptor binding and CB1 receptor agonist-stimulated GTPγS binding in mice

<table>
<thead>
<tr>
<th>Control</th>
<th>Alcohol</th>
</tr>
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<tbody>
<tr>
<td>Bmax (pmol/mg)</td>
<td>12.00 ± 0.3</td>
</tr>
<tr>
<td>Kd (nM)</td>
<td>2.3 ± 0.2</td>
</tr>
<tr>
<td>Fmax (% of basal GTPγS binding)</td>
<td>175 ± 5.25</td>
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</table>

CB1 receptor binding assay was carried out using [3H]CP-55,940 and synaptic plasma membranes (SPM) from alcohol-tolerant mouse brain. Each assay (0.5 ml) containing SPM (100 µg protein) was incubated at 30°C in the presence of [3H] CP-55 940 as described previously (Basavarajappa et al., 1998a). Unlabelled CP-55 940 (1 µM) was used to define non-specific binding. The Bmax (pmol/mg protein) and Kd (nM) were calculated using the Graph Pad prism program. GTPγS binding was done as described before (Basavarajappa and Hungund, 1999b). The data points are mean ± SEM of three experiments done in triplicate. *P < 0.05 (Student’s t-test and non-parametric analyses).

Fig. 1. Development of cellular tolerance to alcohol in cerebellar granular neurons involves the modification of AEA uptake. (A) Dose-dependent inhibition of AEA uptake by various concentrations of acute alcohol. Neurons were pre-incubated at 37°C with various concentrations of alcohol for 5 min and further incubated with [3H] AEA (4 µM) for an additional 3 min. (B) Effect of acute alcohol on AEA uptake in neurons exposed to with or without chronic alcohol. Neurons were exposed to 100 mM alcohol for 72 h (chronic alcohol) and pre-incubated at 37°C with various concentrations of alcohol for 5 min (acute alcohol). AEA uptake was done with [3H] AEA (4 µM) for an additional 3 min as described previously (Basavarajappa et al., 2003). Nonspecific uptake was determined in incubation at 4°C and was subtracted from each data point. Value for control is 0.35 ± 0.05 nmol/10⁶ cells/min. Results are expressed as mean ± SEM of four independent determinations done in duplicates. **P < 0.001 as compared with control.

Table 2. Alcohol tolerance downregulates CB1 receptor binding and CB1 receptor agonist-stimulated GTPγS binding in mice

<table>
<thead>
<tr>
<th>Acute alcohol (mM)</th>
<th>AEA in Cells (nmol/10⁶ cells/min)</th>
<th>AEA Transport (% inhibition)</th>
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<tbody>
<tr>
<td>0</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>50</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>100</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>150</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Graph A shows the dose-dependent inhibition of AEA uptake by various concentrations of acute alcohol. Neurons were pre-incubated at 37°C with various concentrations of alcohol for 5 min and further incubated with [3H] AEA (4 µM) for an additional 3 min. Graph B shows the effect of acute alcohol on AEA uptake in neurons exposed to with or without chronic alcohol. Neurons were exposed to 100 mM alcohol for 72 h (chronic alcohol) and pre-incubated at 37°C with various concentrations of alcohol for 5 min (acute alcohol). AEA uptake was done with [3H] AEA (4 µM) for an additional 3 min as described previously (Basavarajappa et al., 2003). Nonspecific uptake was determined in incubation at 4°C and was subtracted from each data point. Value for control is 0.35 ± 0.05 nmol/10⁶ cells/min. Results are expressed as mean ± SEM of four independent determinations done in duplicates. **P < 0.001 as compared with control.
Dopamine in the NAc (Szabo et al., 1996; Rubino et al., 1997; Zhuang et al., 1998; Breivogel et al., 1999; Di Marzo et al., 2000a). The profound desensitization of cannabinoid-activated signal transduction mechanisms (reduced GTPγS binding) has been shown in the basal ganglia and hippocampus of ∆9-THC administration in rats (∆9-THC tolerant) (Di Marzo et al., 2000a). These results suggest that the observed downregulation of CB1 receptors by chronic alcohol has a dramatic effect on the desensitization of cannabinoid-activated signal transduction, similar to that observed for ∆9-THC or other cannabinoids.

Chronic drug treatment has been shown to change the levels of G-protein and G-protein activity for various G-protein-coupled receptor systems (Szudak et al., 1986; Werling et al., 1988; Wand et al., 1993; Williams et al., 1993; Tabakoff et al., 1995; Traynor and Nahorski, 1995). Various in vitro and in vivo studies have suggested that chronic alcohol treatment leads to reduced sensitivity of adenylate cyclase (Gordon et al., 1986; Charness et al., 1988). A variety of agonists acting at various receptors coupled through Gs to adenylate cyclase have been shown to be reduced by alcohol (Rabin, 1990). Such a modification was suggested to alter the ability of the enzymes to interact with G-proteins and G-protein-coupled receptors (Tabakoff et al., 1995). Regulation of either the G-protein or the G-protein mRNA level by chronic alcohol is also a possibility. Decrease in adenylate cyclase activity (Deitrich et al., 1989; Tabakoff et al., 1995) and a several fold increase in the Gi levels, but no changes in Gsα, have been reported in brains of mice treated with chronic alcohol (Wand et al., 1993). Further studies downstream of the CB1 receptor will be of greater significance in understanding the mechanism involved in the development of tolerance to alcohol.

Dopamine, the CB1 receptor antagonist and voluntary alcohol consumption

There is strong evidence that the dopaminergic system that projects from the ventral tegmental area (VTA) of the midbrain to the nucleus accumbens (NAc) and to other forebrain sites including the dorsal striatum, is the major substrate of reward and reinforcement produced by most drugs of abuse including alcohol (Wise and Bozarth, 1987; Di Chiara and Imperato, 1988; Robbins and Everitt, 1996; Wise, 1996; Koob et al., 1998; Koob and Roberts, 1999; Koob and Le Moal, 2001). It is well established that cannabinoids activate dopaminergic neurons in the VTA (Wise and Bozarth, 1987; Di Chiara and Imperato, 1988; Robbins and Everitt, 1996; Wise, 1996; Tanda et al., 1997; Gessa et al., 1998), resulting in the release of dopamine in the NAc (Szabo et al., 1999). Activation of D2 receptors evokes AEA release in the striatum (Giufrida and Piomelli, 2000). The regulation of dopamine function by cannabinoids is further supported by several biochemical and behavioural studies. In vivo experiments suggest that chronic treatment with D2-receptor antagonists upregulates the CB1 receptor expression in the rat striatum (Mailleux and Vanderhaeghen, 1993). Furthermore, a D2 receptor antagonist has been shown to attenuate the alcohol-induced formation of 2-AG in CGNs (Basavarajappa et al., 2000). In addition, the hyperactivity associated with the postsynaptic D2 receptor activation is accompanied by a dramatic increase in AEA output within the striatum and this effect is potentiated by the CB1 receptor antagonist SR 141716A (Giufrida et al., 1999). Our recent results provide unequivocal evidence that the acute alcohol-induced dopamine release in NAc is mediated by CB1 receptors (Hungund et al., 2003). The acute alcohol-induced increase in dopamine in NAc dialysates in C57BL/6 mice was completely inhibited by pretreatment with the SR 141716A or deletion of the CB1 receptors in mice (CB1 receptor knockout) (Fig. 2; Hungund et al., 2003). Further, SR 141716A blocked alcohol-evoked dopamine release in the shell of the NAc following alcohol administration (Cohen et al., 2002). It should be noted that CB1 receptors are not localized in dopamine cell bodies or in their nerve terminals (Herkenham et al., 1991a; Mailleux and Vanderhaeghen, 1992). It is therefore unlikely that the observed block of alcohol-induced dopamine release by SR 141716A may involve afferent pathways to the VTA. This action may also explain the reducing effects of SR 141716A on alcohol self-administration by indirectly blocking the activation of the mesolimbic dopaminergic transmission (Cohen et al., 2002).

Several studies have shown the inhibition of voluntary alcohol intake by SR 141716A in rodents. SR 141716A has been shown to decrease voluntary alcohol intake in alcohol-preferring C57BL/6 mice (Arnone et al., 1997), in Sardinian alcohol-preferring (sP) rats (Colombo et al., 1998), in alcohol self-administering Long Evans rats (Freedland et al., 2001), and in alcohol-preferring congenic B6.Cb4i5-B13C/Vad and B6.Cb4i5-B14/Vad mouse strains (Hungund et al., 2002). Furthermore, acute administration of the CB1 receptor agonist CP-55,940 increased the motivation to consume alcohol in

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<th>SR141716A + Alcohol</th>
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Fig. 2. Acute alcohol enhances dopamine release in the NAc of CB1 receptor wild-type but not in knockout male mice. CB1 receptor WT and KO male mice received an i.p. injection of saline followed by alcohol (1.5 g/kg). Dialysate samples were collected every 20 min before and after each injection. Data from wild-type (n = 6) and knockout (n = 6) mice are represented at 20 min peak value from our previous data (Hungund et al., 2003). Wild-type and knockout mice received an i.p. injection of vehicle followed by CB1 receptor antagonist SR 141716A (3 mg/kg) 40 min prior to the administration of alcohol (1.5 g/kg). Dialysate samples were collected every 20 min before and after each injection. Data from wild-type mice (n = 6) and knockout mice (n = 3) are represented at 20 min peak value from our previous data (Hungund et al., 2003). ***P < 0.001 (GLM repeated measures ANOVA).
Wistar rats and this effect was completely prevented by pretreatment with the CB₁ receptor antagonist SR 141617A (Gallate et al., 1999; Gallate and McGregor, 1999). An acute dose of SR 141716A completely abolished the alcohol deprivation effect (i.e. the temporary increase in alcohol intake after a period of alcohol withdrawal) in sP rats (Serra et al., 2002). Acute administration of CB₁ receptor agonists WIN-55,212–2 and CP-55,940 significantly stimulated voluntary alcohol consumption in alcohol-prefering sP rats and this was prevented by SR 141716A (Colombo et al., 2002). None of these studies investigated the possible involvement of dopamine in CB₁ receptor-regulated voluntary alcohol intake in these animals.

The adaptive changes noted in the endocannabinoid system after chronic alcohol treatment may be important for the development of alcohol-seeking behaviour and further research is required to establish this phenomenon. The available evidence for the participation of the cannabino-ergic system in alcohol drinking behaviour is derived from the observed differences in CB₁ receptor function in two genetic strains of alcohol-preferring C57BL/6 and alcohol-avoiding DBA/2 mice. In this study, we found that C57BL/6 mice have a significantly lower level of CB₁ receptor binding sites and higher affinity for [³H]CP-55,940 than DBA/2 mice (Hungund and Basavarajappa, 2000b). Interestingly, the significantly higher levels of CB₁ receptors found in DBA/2 mice are less coupled to G-proteins as shown by GTPγS binding assay compared with C57BL/6 mouse strains (Table 3; Basavarajappa and Hungund, 2001), suggesting the participation of these receptors in controlling voluntary alcohol consumption. Thus, genetically determined differences in the activities of distinct components of the endogenous cannabino-ergic system under basal conditions or in response to alcohol exposure may exist between alcohol-preferring and alcohol-avoiding animals and may be partially responsible for the differences in their voluntary alcohol intake. This hypothesis was further examined using genetically modified CB₁ receptor knockout mice. Genetics and CB₁ receptor aspects of alcoholism are covered elsewhere in this special issue (Lallemand and De Witte, 2004).

CONCLUSION

Over the past seven years, remarkable advances have been made towards our understanding of the role played by the endocannabinoid system in the development of alcohol tolerance and alcohol-drinking behaviour. These studies have provided strong evidence that CB₁ receptors and the endocannabinoid system serve as an attractive therapeutic target for the treatment of alcohol tolerance and alcohol-related disorders. The data reviewed here provide convincing evidence that alcohol tolerance involves the downregulation of the CB₁ receptor and its function. The observed neuro-adaptation may be due to increased accumulation of the endocannabinoids AEA and 2-AG. Treatment with the CB₁ receptor antagonist SR 141716A led to reduced consumption of alcohol in rodents and activation of the same endogenous cannabinoid systems by the CB₁ receptor agonist promoted alcohol craving, which may be related to the change in the levels of dopamine in the NAc. Further, reduced alcohol intake by the CB₁ receptor knockout mice is consistent with our previous observation that significantly lower levels of functional CB₁ receptors are found in the alcohol-avoiding DBA/2 mouse strain compared with the alcohol-preferring C57BL/6 mouse strain. These observations suggest the involvement of the CB₁ receptors in controlling voluntary alcohol consumption and the involvement of the endocannabinoid system in the development of alcohol tolerance. However, further studies are necessary to unfold the exact mechanism by which alcohol exerts its pharmacological and behavioural effects through the endocannabinoid system. The investigation of the detailed signalling cascade for the actions of both endocannabinoids and CB₁ receptors will be of great value in understanding their physiological and functional role in several neurological disorders, voluntary alcohol intake and alcohol craving, including the behavioural neuroadaptation to alcohol. Such studies may also lead to the development of endocannabinoid signalling-targeted drugs, which may help to reduce both alcohol intake and alcohol craving. These results suggest that the cannabinoid antagonist, SR 141716A, may be useful as a potential therapeutic agent in alcohol dependence.

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| Table 3. Changes in CB₁ receptor binding and its agonist-stimulated GTPγS binding in the brain of C57BL/6 and DBA/2 mouse strains |
|----------------|----------------|----------------|
|                | C57BL/6        | DBA/2          |
| Bmax (pmol/mg protein) | 0.662 ± 0.03   | 0.883 ± 0.08*  |
| Kd (nM)           | 0.68 ± 0.2     | 2.21 ± 0.56*   |
| G-protein Bmax (pmol/mg protein) | 12.43 ± 0.64   | 9.46 ± 0.98*   |
| Emax (% of basal GTPγS binding) (CP-55,940) | 129.2 ± 1.82 | 121.1 ± 0.71* |

The CB₁ receptor binding assay was carried out using [³H] CP-55,940 and plasma membranes (PM) from brain of C57BL/6 and DBA/2 mouse strains. Each assay (0.5 ml) containing PM (75 µg protein) was incubated at 30°C in the presence of [³H] CP-55,940 as described previously (Basavarajappa et al., 1998a; Hungund and Basavarajappa, 2000b). Unlabelled CP-55,940 (10 µM) was used to define non-specific binding. CB₁ receptor agonist-stimulated [³H]-GTPγS binding assay was carried out using [³H]-GTPγS and PM from brain of C57BL/6 and DBA/2 mouse strains. Each assay (0.5 ml) containing PM (100 µg/ml) was incubated at 30°C in the presence or absence of CP-55,940, GDP (100 µM) as described previously (Basavarajappa and Hungund, 1999b, 2001). Unlabelled GTPγS (10 µM) was used to define non-specific binding. The data points are mean ± SEM of three experiments done in triplicate. The Bmax (pmol/mg protein) and Kd (nM) were calculated using the Graph Pad prism program. *P < 0.05 (Student’s t-test and non-parametric analyses).


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