

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-arachidonylglycerol 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 been 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 SR 141716A 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 amide 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, basal 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; Sugiura *et al.*, 1995), 2-arachidonylglycerol 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*-arachidonylphosphatidylethanolamine (N-ArPE), which is believed to originate from the transfer of arachidonic acid (AA) from the sn-1 position of 1,2-sn-di-arachidonylphosphatidylcholine to phosphatidylethanolamine,

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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 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

Table 1. Chronic alcohol enhances endocannabinoid levels in neuronal cells

	Control	Alcohol
AEA		
Basal	100.0 ± 5.8	138.3 ± 5.7*
SR 141716A (1 µM)	91.9 ± 10.9	92.4 ± 6.2*
PTX (100 ng/ml)	106.8 ± 8.4	115.7 ± 18.1*
2-AG		
Basal	100.0 ± 17.0	165.0 ± 23.5*
SR 141716A (1 µM)	104.7 ± 9.1	109.5 ± 14.0*
PTX (100 ng/ml)	110.3 ± 5.0	118.8 ± 17.1*

SK-N-SH cells (AEA) and CGNs (2-AG) were labelled with [³H] AA (1 µCi/ml) in 0.1% FBS DMEM or BEM (without FCS) for 5 h and then co-treated with SR 141716A (1 µM) or PTX (100 ng/ml) and with or without alcohol (100 mM). The [³H] AEA and [³H] 2-AG were extracted from media and cells and separated on TLC as described previously (Basavarajappa and Hungund, 1999a; Basavarajappa *et al.*, 2000). Data are expressed as percentage of control. Control values are 5700 ± 400 d.p.m./mg protein for AEA and 40 550 ± 7468 d.p.m./mg protein for 2-AG. Each value represents the mean ± SEM (*n* = 9). **P* < 0.05 (ANOVA).

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.*, 1999; 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

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 ethanolamide derivative of AA. Arachidonic acid is derived from membrane lipids by selective activation of Ca^{2+} -dependent PLA2, which releases the Sn-2-arachidonyl moiety from the membrane PL, phosphatidylcholine (PC) (Basavarajappa *et al.*, 1997, 1998b). 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 phenylmethylsulfonyl fluoride (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 (Sugiura *et al.*, 2002). In our recent study, glutamate, which is also known to stimulate the formation of *N*-acylethanolamides (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 $[\text{Ca}^{2+}]_i$ after 4 days of exposure to alcohol (Iorio *et al.*, 1992), suggesting that the glutamate-induced increase in intracellular Ca^{2+} 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 Ca^{2+} 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 (Giuffrida *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 is the treatment of problems associated with drugs of abuse, including alcohol.

AEA signalling at the cannabinoid CB_1 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; Rakhshan *et al.*, 2000). Co-localization of both FAAH and CB_1 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 CB_1 receptor since CB_1 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 CB_1 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 B_{max} of CB_1 receptors and inhibited the ability of the CB_1 receptor agonist to stimulate $\text{GTP}\gamma\text{S}$ 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 CB_1 receptor signalling system by chronic alcohol may also result from

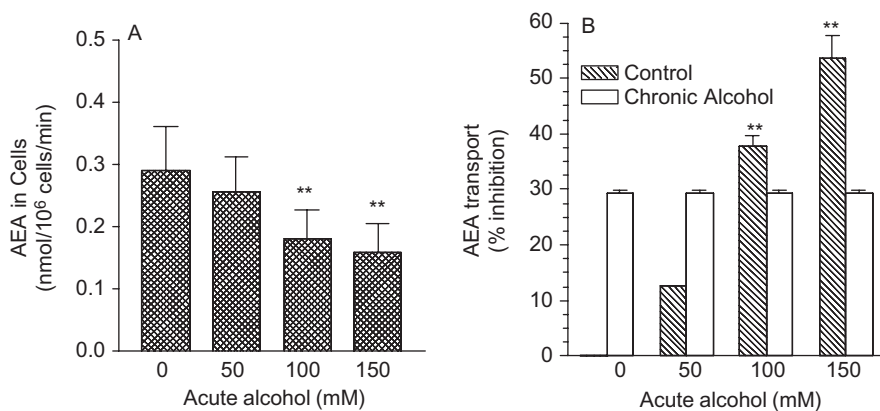


Fig. 1. Development of cellular tolerance to alcohol in cerebellar granular neurons involves the modification of AEA uptake. (A) Dose-dependent inhibition of AEA uptake by various concentrations of acute alcohol. Neurons were pre-incubated at 37°C with various concentrations of alcohol for 5 min and 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 by 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

	Control	Alcohol
B_{max} (pmol/mg protein)	12.00 ± 0.3	7.00 ± 0.3*
K_d (nM)	2.3 ± 0.2	3.0 ± 0.3
E_{max} (% of basal GTPγS binding) (CP-55,940)	175 ± 5.25	150 ± 8.14*

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 B_{max} (pmol/mg protein) and K_d (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).

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 caudate-putamen (Cpu), the ventromedial nucleus of the hypothalamus (VMN), and CA1 and CA2 fields of the hippocampus (Ortiz *et al.*, 2004). It was shown that chronic alcohol exposure of rats was not effective in altering either CB₁ receptor binding (measured by [³H]-CP-55,940 autoradiography) or mRNA levels (measured by *in situ* hybridization) in the regions of the brain (Gonzalez *et al.*, 2002a). The reason for this inconsistency is not known. However, it should be noted here that in the studies reported by Gonzalez *et al.* (2002a) and Ortiz *et al.* (2004) rats were exposed to chronic alcohol using alcohol liquid diet, whereas in our studies (Basavarajappa *et al.*, 1998a) mice were exposed to chronic

alcohol 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 Zaechelein, 1983; Gatch and Lal, 1999; Rasmussen *et al.*, 2002). Various agonists acting at various receptors coupled through Gs to adenylate cyclase have been shown to be reduced by alcohol (Rabin, 1990). Such a change was suggested to modify the ability of the enzymes to interact with G-proteins and G-protein-coupled receptors. There have also been studies showing a decrease in adenylate cyclase activity and an increase in the Gi levels, but no change in Gα was observed 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 ('A' current) (Deadwyler *et al.*, 1995; Mackie *et al.*, 1995) and inhibition of Ca²⁺ current by a voltage-gated N-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

(Table 2; Basavarajappa and Hungund, 1999b). It has also been shown that in addition to the CB₁ receptor modification by CB₁ receptor agonists, downregulation of G-protein expression and CB₁ receptor agonist-stimulated GTPγS binding was also observed in the central nervous system of the rat (Sim *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 Δ⁹-THC-tolerant rats (Sim *et al.*, 1996). This was also noted in most regions of the brain except the limbic forebrain, analysed after 8 days of daily Δ⁹-THC administration in rats (Δ⁹-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 Δ⁹-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 (Suzdak *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 Gα_s, 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 (Giuffrida 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 (Giuffrida *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-β/13C/Vad and B6.Cb4i5-β14/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

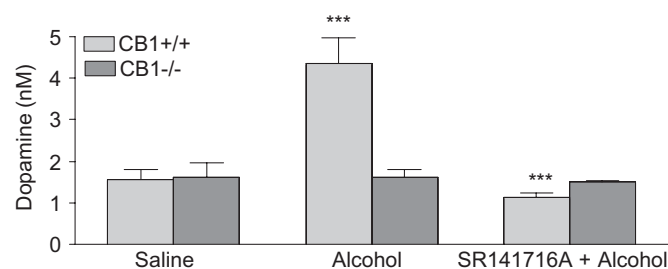


Fig. 2. Acute alcohol enhances dopamine release in the NAc of CB₁ receptor wild-type but not in knockout male mice. CB₁ receptor WT and KO male mice received an i.p. injection of saline followed by alcohol (1.5 g/kg). Dialysate samples were collected every 20 min before and after each injection. Each data point represents the average DA level ± SEM. 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-preferring 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 cannabinoid 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 cannabinoid 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 (Lallemant 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 neuroadaptation 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

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
B_{\max} (pmol/mg protein)	0.662 ± 0.03	0.883 ± 0.08*
K_d (nM)	0.68 ± 0.2	2.21 ± 0.56*
G-protein B_{\max} (pmol/mg protein)	12.43 ± 0.64	9.46 ± 0.98*
E_{\max} (% 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 B_{\max} (pmol/mg protein) and K_d (nM) were calculated using the Graph Pad prism program. * $P < 0.05$ (Student's *t*-test and non-parametric analyses).

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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