Regulation of brain anandamide by acute administration of ethanol

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INTRODUCTION

Several research lines have established a role for both the eCB (endocannabinoid) AEA (arachidonylethanolamide or anandamide) and the CB1 (cannabinoid 1) receptor in ethanol-dependence [1–3]. While administration of CB1 receptor agonists promotes ethanol intake [4], the administration of a CB1 receptor antagonist decreases ethanol self-administration [5], especially in animals with a history of ethanol-dependence [6] or in alcohol-prefering rat lines [7]. Following these findings, CB1-receptor-knockout mice were found to display decreased ethanol preference, a phenotype that resembled that of wild-type littermates treated with a CB1 receptor antagonist [3].

Molecular studies have shown that chronic ethanol administration is associated with an increased formation of both AEA and its membrane precursor N-arachidonoyl phosphatidylethanolamine [8]. Chronic alcohol exposure also resulted in stimulation of 2-arachidonoylglycerol, a second eCB [9]. Animal studies also revealed that chronic exposure to ethanol downregulated CB1 receptors in the brain [10]. Finally, a gene-screening study has identified the CB1 receptor as one of the genes whose expression is permanently affected by serial cycles of ethanol-dependence and withdrawal [11]. These data indicate a role for the eCB system as a relevant contributor to alcoholism. Human genetic studies support this experimental hypothesis, since a linkage between clinical forms of alcoholism and polymorphisms and/or mutations of the genes encoding either the CB1 receptor [12,13] or FAAH (fatty acid amidohydrolase) [14], the enzyme responsible for AEA inactivation [15], have been described.

Despite the extensive information on the effects of chronic exposure to alcohol, no information is available on the acute effects of ethanol on AEA formation and degradation. We have addressed this issue by analysing the time course of the effects of acute ethanol on AEA and the related mediator PEA (palmitoylethanolamide) in the rat brain, and that this effect is not derived from increased fatty acid ethanolamide degradation.

Key words: alcohol, anandamide, arachidonylethanolamide (AEA), cannabinoid, fatty acid amidohydrolase (FAAH), hippocampus.
MATERIALS AND METHODS

Animals

Male Wistar rats (Charles River Laboratories), weighing 350±50 g, were housed individually with food and water available ad libitum. Animals were kept in a standard 12 h light/12 h dark photoperiod (lights on 08:00 h–20:00 h). They were handled daily for 1 week before starting the experiments, and they received intraperitoneal saline injections once a day in the 3 days that preceded the experimental administration of ethanol. All animal procedures met the National Institutes of Health guidelines for the care and use of laboratory animals, and the European Communities directive 86/609/EEC regulating animal research.

Chemicals

Unlabelled AEA and PEA standards and [3H]-labelled AEA and PEA were synthesized as described in [30]. 1,2-Di[3H]palmitoyl-sn-glycerophosphocholine (108 mCi/mmol) was purchased from Amersham Biosciences, and [1−3H]ethanolamine hydrochloride (60 Ci/mmol) was from American Radiolabeled Chemicals.

Vehicle and ethanol administration: blood and tissue sampling

Ethanol was dissolved in sterile 0.9% (w/v) saline (NaCl) before intraperitoneal injection. Rats received either 15% (w/v) ethanol solution (0.75, 2 or 4 g/kg of body mass) or a similar volume of a 0.9% saline solution (control group). In order to avoid potential circadian variations on eCB levels, as described previously [31], all the experiments took place between 09:00 h and 13:00 h during the light phase of the cycle. Since it has been described that restraint stress may affect brain AEA levels in specific brain areas [32], we first explored the potential effects of the injection procedure on the brain levels of this eCB. To this end, we analysed whether a single saline injection affected AEA concentrations in the striatum nucleus accumbens, cerebellum and dorsal hippocampus. A group of injection-habituated animals was anesthetized with methoxyflurane (Schering-Plough) 0, 5, 30 or 60 min after the injection of 0.9% saline (1 ml/kg of body mass). The brains were collected after rapid decapitation and were frozen immediately in solid CO2. Dissection of frozen brains was performed at −10 °C. Brains were placed in acrylic rat brain matrices, and 2-mm-thick slices were obtained using brain matrix razor blades. The target brain regions were collected using a scalpel (cerebellum and dorsal hippocampus) or a sample corer (nucleus accumbens). The nucleus accumbens, dorsal hippocampus and cerebellum were dissected in 2-mm-thick frozen coronal cuts, corresponding to plates 9–13 (nucleus accumbens, dorsal hippocampus and cerebellum) and 26–36 (dorsal hippocampus) and 56–65 (cerebellum) of the atlas of Paxinos and Watson [33].

In order to evaluate the effects of ethanol on blood and brain eCB content, two more experiments were performed. For blood and visceral sampling, a group of animals was anesthetized with methoxyflurane 0, 45, 90 or 240 min after the injection of ethanol. Blood (2 ml) was collected from the heart of the animal with a syringe filled with 1 ml of Krebs–Tris buffer (1.36 mM NaCl, 6 mM KCl, 1.2 mM MgCl2, 2.5 mM CaCl2, 10 mM glucose and 20 mM Trizma base, pH 7.4). Blood samples were centrifuged at 800 g for 10 min in Accuspin tubes (Sigma), and plasma layers were used for monitoring plasma AEA as described in [30]. Samples were obtained from perirenal fat, liver and ileum and were frozen immediately in solid CO2. A second group of animals were killed following the above-described protocol at 0, 45, 90 or 240 min after the injection of ethanol. The brains were collected and were frozen immediately in solid CO2. The nucleus accumbens, dorsal hippocampus and cerebellum were dissected as described above.

Plasma ethanol levels

Plasma ethanol levels were measured enzymatically using a commercial kit (Sigma). Assays were run following the manufacturer’s instructions.

HPLC–MS analyses of tissue contents of acylethanolamides

AEA and PEA were solvent-extracted from plasma, brain areas (nucleus accumbens, dorsal hippocampus and cerebellum) and peripheral tissues (perirenal fat, liver and ileum), fractionated by column chromatography and quantified by HPLC–MS with an isotope-dilution method [30,34].

Real-time PCR for relative quantification of CB1 receptor, FAAH, NAPE-PLD and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) mRNA expression

Total RNA from nucleus accumbens, hippocampus and cerebellum was obtained using TRIzol® reagent (Gibco BRL Life Technologies) according to the manufacturer’s instructions. All RNA samples showed A260/A280 ratios between 1.8 and 2.0. First-strand synthesis from each sample was done using random hexamer primer and MMLV (Moloney murine leukaemia virus) reverse transcriptase (Roche Applied Science) according to the manufacturer’s instructions. Negative-control reactions omitted reverse transcriptase. Resulting cDNAs were used as the template for real-time quantitative PCR, which was performed using the LightCycler instrument (Roche) with the SYBR Green I detection format.

The following primers were used (accession numbers from the GenBank® database are given in parentheses): CB1 receptor (NM_012784) forward, 5′-AGACCTCTCTCTACGCTGGCTCG-3′, and reverse, 5′-GTACACGGATGCCCAGCTGCT-3′ (314 bp product); FAAH (U72497) forward, 5′-GGTACAGATTGGAGA- GCTCTC-3′, and reverse, 5′-GGAGTTTACTGCAATGAAA-GC-3′ (344 bp product); NAPE-PLD (AB112351) forward, 5′-GGAGTTTATGAAGCCAGT-3′, and reverse, 5′-ACTCTCGTGTCCTCAGATG-3′ (223 bp product); GAPDH forward, 5′-GGGTGATGCTCTGCTGAT-3′, and reverse, 5′-AGTCTTGGTGCTAGCCAAGT-3′ (272 bp product); and GUS (β-glucuronidase) (NM_017015) as an endogenous control forward, 5′-TCTGTACACCAACCTACCAC-3′, and reverse, 5′-GCCATCTCATCAGGAGAC-3′ (146 bp product). Primers were obtained from Prologo.

Quantification was carried out on the basis of standard curves run simultaneously with samples [35]. CB1 receptor, FAAH, NAPE-PLD, GADPH and GUS standards were generated by PCR-amplification from control samples. The PCR product was run on 1% agarose gel electrophoresis for checking the fragment size and the absence of other contaminant fragments, quantified by measuring the A260, and serially diluted to 10−5 pg/µl. Several 10-fold dilutions (10−1–10−5) were checked for optimal cycling using the LightCycler and three of them were selected for standard curves, taking into consideration mRNA abundance. Each reaction was run in duplicate and contained 3 µl of cDNA template, 3 mM MgCl2, and 0.5 µM of primers in a final reaction volume of 20 µl. Cycling parameters were 95 °C for 10 min to activate DNA polymerase, then 30–40 cycles of denaturation at 95 °C for 10 s, annealing for 10 s (CB1 receptor, 62 °C; FAAH, 41 °C; NAPE-PLD, 57 °C; GADPH, 66 °C; GUS, 61 °C) and a final extension step of 72 °C for 16 s in which fluorescence was acquired. Melting-curve analyses were performed to ensure that
only a single product was amplified. Data for CB1 receptor, FAAH, NAPE-PLD and GAPDH were normalized for expression of GUS.

Preparation of rat tissue subcellular fractions

Tissues were homogenized in 50 mM Tris buffer, pH 8, containing 0.32 M sucrose. Homogenates were first centrifuged at 1000 g for 5 min, the pellet was then discarded and the supernatant was centrifuged at 45000 g for 30 min. The pellets obtained were solubilized at 0–4 °C in Tris buffer. The protein content in the membrane fraction was measured using the Bradford method. All tissue samples and membrane fractions were stored at −70 °C until use.

Enzymatic determinations

We assayed membrane-bound FAAH activity using [3H]AEA (arachidonoyl-[1-3H]ethanolamide) as a substrate, and measuring metabolized [3H]AEA (as [3H]ethanolamine) in the aqueous phase after chloroform extraction, as described in [34,35]. Microsomal NAT activity assays were performed using 1,2-di[14C]palmitoyl-sn-glycerophosphocholine as substrate [36]. Both enzymatic assays were run under conditions that were linear in time and protein concentration.

In vivo microdialysis of AEA and glutamate

Drug-naive male Wistar rats (n = 11) were anaesthetized using 1–2% isoflurane vapour and implanted with microdialysis guide cannula (20-gauge) (SciPro) aimed at the nucleus accumbens shell (from bregma: anteroposterior +1.6 mm, midline ±0.8 mm, and dorsoventral 5.7 mm from dura [33,37]). A period of 7 days of post-operative recovery was allowed before testing. On the day before microdialysis, animals were anaesthetized with 1–2% isoflurane vapour and a microdialysis probe (2 mm polyether-sulfone membrane, 15 kDa molecular-mass cutoff, SciPro) was inserted and secured to the previously implanted guide cannula. Probes were perfused overnight with aCSF (artificial cerebrospinal fluid; 145 mM NaCl, 2.8 mM KCl, 1.2 mM MgCl2, 1.2 mM CaCl2, 5.4 mM d-glucose and 0.25 mM ascorbic acid, pH 7.2–7.4) at a rate of 0.2 µl/min. The following morning, the perfusate was switched to an aCSF containing 30% (w/v) HB-β-CD (hydroxypropyl-β-cyclodextrin) and the flow rate was increased to 0.6 µl/min. Inclusion of HB-β-CD in the perfusate provides a substantial increase in the dialysis recovery of eCBs (similar to the findings by Walker et al. [38]). Following a 90 min equilibration period, dialysate samples were collected at 10 min intervals for a 40 min baseline period, for 30 min following vehicle injection (ethanol/Emulphor®/saline, 1:1:18, by vol.; 1 ml/kg of body mass) and finally for 120 min after intra-peritoneal 20% (w/v) ethanol administration (0.75 or 2 g/kg of body mass). Dialysate samples were frozen immediately following collection and stored at −80 °C until analysis for eCB content using LC–MS as described below.

A second group of male Wistar rats (n = 5) were surgically implanted with microdialysis guide cannulae aimed at the nucleus accumbens for the analysis of ethanol-induced alterations in interstitial glutamate levels. The same protocol as described above was followed, with the exception that the dialysis perfusate did not contain HB-β-CD (this additive is not necessary for the recovery of monoamines and amino acids) and a higher perfusate flow rate was employed (1 µl/min) to allow sample splitting for the monoamine and amino acid analyses (see below).

LC-MS analysis of dialysate eCB content

Dialysate levels of AEA were determined using LC–electrospray ionization MS. Microdialysate aliquots (5 µl) were spiked with 5 µl of 100 nM S-2 methanandamide [(S)-(−)-arachidonoyl-2′-hydroxy-1′-propylamide] and loaded on to a pre-column (0.5 mm × 2.5 mm, Haisil HL C18, 5 µm particle size) (Higgins Analytical) using a 30% (v/v) methanol mobile phase delivered at 70 µl/min. Following a 2 min wash period, mobile-phase flowthrough the pre-column was reversed via a switching valve and the eCBs were delivered to a 0.3 mm × 50 mm microbore analytical column (Haisil HL C18, 3 µm particle size) (Higgins Analytical) using an isocratic mobile phase consisting of 70% (v/v) methanol delivered at 5 µl/min. The analytical column eluent was delivered via a nanoelectrospray interface into the mass spectrometer (Agilent 1100MSD) that was run in positive selected ion monitoring mode to maximize sensitivity. Similar to findings by others [30], we found that sodium adducts of these molecules provide greater sensitivity than do their protonated forms. The following mass/charge (m/z) ratios were used: AEA, 370.3 (M + 1Na); S-2 methanandamide, 384.3 (M + 1Na). External calibration curves were constructed from a minimum of three standard concentrations (each run in duplicate) and were generated daily. Under these conditions, the limits of quantification were approx. 0.1 nM.

Analysis of dialysate glutamate

Microdialysis samples were split into two 5 µl aliquots for quantification of glutamate content by CE–LIF (capillary electrophoresis coupled with laser-induced fluorescence detection). Specific details of these analyses have been published previously [39].

Statistics

Statistical significance was assessed by one-way ANOVA. Following a significant F value, post-hoc analysis (Student–Newman–Keuls) was performed. Calculations were made using the BMDP statistical package.

RESULTS

Acute injection of saline does not affect brain AEA levels

The acute injection of saline did not affect brain AEA levels in the nucleus accumbens, cerebellum or dorsal hippocampus (Table 1). Since saline injection in habituated animals is a mild stressor, the lack of effects of this procedure on brain eCB content is in agreement with previous reports [32] which described a decrease in AEA only after severe stress induced by restraint, and only in the amygdala (and not in the cerebellum or the forebrain, including the nucleus accumbens).

<table>
<thead>
<tr>
<th>Brain section</th>
<th>Time after injection (min)</th>
<th>AEA (pmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleus accumbens</td>
<td>0</td>
<td>119 ± 20</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>98 ± 40</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>82 ± 29</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>0</td>
<td>16.5 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>14.7 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>26.7 ± 1.9</td>
</tr>
<tr>
<td>Dorsal hippocampus</td>
<td>0</td>
<td>137 ± 51</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>134 ± 63</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>119 ± 41</td>
</tr>
</tbody>
</table>
Figure 1  Time course of the effects of acute intraperitoneal administration of ethanol on plasma levels of ethanol and AEA

The effects of ethanol (4 g/kg of body mass) on plasma levels of (A) ethanol and (B) AEA were determined. Results are means ± S.E.M. for at least five determinations per group. No significant differences were found between samples. N.D., not determined.

Table 2  Effects of ethanol on AEA levels in various tissues

Effects of acute intraperitoneal administration of ethanol (4 g/kg of body mass) on AEA levels (pmol/g of tissue) in perirenal fat, liver and small intestine were measured 0, 45, 90 and 240 min after injection. Results are means ± S.E.M. for five to eight determinations per group. *P < 0.01 compared with the zero-time group.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Time after injection (min)</th>
<th>AEA (pmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>45</td>
</tr>
<tr>
<td>Fat</td>
<td>16.2 ± 1.7</td>
<td>11.9 ± 0.9</td>
</tr>
<tr>
<td>Liver</td>
<td>3.7 ± 1.8</td>
<td>3.7 ± 1.8</td>
</tr>
<tr>
<td>Small intestine</td>
<td>33.9 ± 13.2</td>
<td>63.4 ± 23.5</td>
</tr>
</tbody>
</table>

Acute ethanol decreases plasma AEA levels

The acute intraperitoneal administration of 4 g of ethanol/kg of body mass led to a rapid rise in plasma ethanol levels, which then remained stable for 4 h after the administration (Figure 1A). Clearance of plasma ethanol was evident 8 h after the injection and was complete within the first 24 h. Plasma AEA levels were affected after acute ethanol exposure (Figure 1B): AEA was not detectable in plasma 45 min after acute ethanol exposure, returned to normal levels within the following 45 min, peaked at 4 h and returned to control levels at 8 h (1.3 ± 0.6 pmol/ml; n = 8) and 24 h (0.7 ± 0.5 pmol/ml; n = 8). In peripheral tissues, ethanol decreased AEA levels in perirenal fat, but resulted in a marked increase in levels in the small intestine and liver 90 min after the injection (Table 2).

On the basis of both the presence of stable ethanol levels and on the changes in plasma AEA, we selected times of 0, 45, 90 and 240 min for the biochemical analysis of both AEA and PEA and the enzymatic activities of FAAH and NAT in the brain.

Acute ethanol administration decreases AEA and PEA in the nucleus accumbens, cerebellum and hippocampus

Acute injection of ethanol decreased AEA levels in the nucleus accumbens (Figure 2A), cerebellum (Figure 2B) and hippocampus (Figure 2C). This effect was observed in all brain areas 90 min after ethanol administration. In the hippocampus, the reduction of AEA levels was observed at all time points. Similarly, ethanol decreased PEA in the nucleus accumbens (Figure 3A) 45 min after its injection, in the cerebellum at 45 and 90 min (Figure 3B) and in the hippocampus at all time points studied (Figure 3C).

Acute ethanol administration does not increase FAAH activity

In order to investigate whether the decrease in the levels of AEA and PEA observed after acute ethanol might be derived from changes in the activity of the enzyme involved in the degradation of these acylethanolamides, FAAH, we tested its activity in membrane fractions obtained from animals acutely exposed to ethanol. FAAH activity was not affected by ethanol in either membranes from nucleus accumbens or from cerebellum (Figures 4A and 4B). However, FAAH activity was decreased 45 min after alcohol administration in the hippocampus, normalizing thereafter. Additional studies performed in vitro using cerebellar membranes indicated that FAAH activity was not affected by the presence of ethanol in the buffer at concentrations...
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Figure 3  Time course of the effects of acute intraperitoneal administration of ethanol on tissue levels of PEA

The effects of ethanol (4 g/kg of body mass) on levels of PEA in (A) nucleus accumbens, (B) cerebellum, and (C) dorsal hippocampus. Results are means ± S.E.M. for at least five determinations per group. *P < 0.01 compared with the zero-time group.

Acute ethanol administration does not affect the expression of CB1 receptor, FAAH and NAPE-PLD mRNA in the hippocampus or the cerebellum

Since we observed that ethanol decreased FAAH activity in the hippocampus, we monitored the expression of FAAH mRNA as well as that of NAPE-PLD, the recently cloned enzyme that cleaves AEA from its membrane precursor (Figure 5). Any of the eCB-related genes were affected by acute ethanol administration either at 2 or at 12 h after the injection. However, GADPH expression was clearly increased after acute alcohol exposure. This pattern was observed also in both the hippocampus and the cerebellum. These results point against an activation of degradation as the main origin of the ethanol-induced decrease of AEA in the brain.

Acute ethanol administration does not affect NAT activity

The activity of the enzyme involved in the synthesis of the AEA precursor N-arachidonoyl phosphatidylethanolamine, NAT, was not affected in cerebellar membranes after acute intraperitoneal administration of ethanol (NAT activity was 452 ± 108 pmol/min per mg at 0 min, 449 ± 104 pmol/min per mg at 45 min, 687 ± 234 pmol/min per mg at 90 min, and 385 ± 122 pmol/min per mg at 120 min n = 5–8). We could not measure NAT activity on an individual hippocampus because the technique requires pooling of various samples in order to reach a sufficient amount of protein.

Acute ethanol administration decreases AEA in the nucleus accumbens

Baseline dialysate AEA concentrations were 2.03 ± 0.5 and 2.12 ± 1.1 nM for the 0.75 g/kg (n = 6) and 2.0 g/kg (n = 5) ethanol groups respectively. Although vehicle injection produced no significant alteration in dialysate AEA levels, intraperitoneal ethanol administration led to a significant and dose-dependent decrease in dialysate AEA concentrations (F(1,141) = 25.094, P < 0.001) (Figure 6A). Administration of 0.75 g of ethanol/kg of body mass produced a subtle and non-significant decrease in dialysate AEA concentrations which was evident within 40 min of administration and persisted at approx. 60% of pre-ethanol baseline levels for the remainder of the 120 min post-ethanol sample-collection period.

Acute ethanol administration decreases glutamate in the nucleus accumbens

The baseline dialysate glutamate level in the same samples as above was 602 ± 21 nM. Glutamate levels in the dialysate samples were significantly reduced following 2 g of ethanol/kg
of body mass ($F_{1,12} = 4.679; P < 0.0001$) which was evident within 30 min of administration and persisted at approx. 70% of pre-ethanol baseline levels for the remainder of the 120 min post-ethanol sample-collection period (Figure 6B).

**DISCUSSION**

These results suggest, first, that acute exposure to ethanol affects AEA formation in the brain and peripheral tissues, and, second, that this effect is not dependent on ethanol-induced activation of AEA degradation by FAAH. The effects on AEA were replicated for a second acylethanolamide, PEA, that shares with AEA biochemical pathways for synthesis or degradation, suggesting a common mechanism for the decrease of both acylethanolamides. In vivo microdialysis experiments suggest that the effect on AEA release might be dependent on the inhibitory actions of ethanol on glutamate release, one of the main triggers for AEA formation.

Ethanol induced a marked decrease in the formation of AEA in the brain, plasma and adipose tissue, which was time-limited to the first 90 min of study (Figures 1 and 2). Opposite effects were observed in the liver and, especially, in the small intestine, where a marked increase in AEA release was also observed 90 min after ethanol administration. These effects suggest the existence of tissue-selective eCB targets for ethanol. Increases in AEA levels in tissues may be dependent either on changes in the synthesis of AEA precursor $N$-arachidonoyl phosphatidylethanolamine, or on the cleavage of $N$-arachidonoyl phosphatidylethanolamine species by receptor-activated PLD (phospholipase D), or by blockade of FAAH-dependent enzymatic degradation of AEA [28,15,27]. Since there was no activation of FAAH by ethanol in vivo or in vitro, and since initial observations indicated no changes in NAT activity (Table 2), we propose that the decrease in AEA and PEA release observed may be dependent on reduced receptor-activated NAPE-PLD activity, a major source for the release of acylethanolamides [37,39,40]. Acute ethanol administration might then operate through the modulation of neurotransmitters capable of activating specific receptors coupled to NAPE-PLD. This mechanism may not require a substantial modification of NAPE-PLD production because the inhibition of the release of neurotransmitters will result solely in reduced eCB production. Following this rationale, we observed that the expression of this synthetic enzyme is not affected 2 h after the administration of ethanol, although further research is needed to clarify whether chronic administration of alcohol might affect its expression and activity, leading to the increased production of eCB described in animal models of alcoholism.

Several neurotransmitters may contribute to a decrease in PLD-mediated NAPE breakdown and the subsequent lowering of AEA and PEA levels. First, acute ethanol may decrease
glutamate, and acetylcholine-dependent PLD activation through its well-described inhibitory effects on presynaptic release of these neurotransmitters [25,26]. In this regard, the microdialysis experiments confirm this hypothesis, since we found parallel decreases in extracellular levels of both AEA and glutamate in the nucleus accumbens (Figure 6) after acute ethanol exposure. Chronic ethanol exposure will result in the development of tolerance and ultimately to increased release of both transmitters [41,42], which will contribute to the enhanced AEA formation associated with chronic ethanol exposure [2]. Secondly, ethanol may directly inhibit glutamate NMDA (N-methyl-D-aspartate) and metabotropic receptors involved in PLD-dependent AEA release, leading to a decrease in NAPE breakdown [19,25,39]. Although there is direct evidence that ethanol might disrupt PLD-dependent signalling, acting as a metabolic substrate that shifts PLD activity to the production of the phospholipid phosphatidylethanolamine [43], this hypothesis cannot be used to explain the effects observed in the present study, since recent reports indicate that NAPE-PLD does not use ethanol as a substrate [29].

An additional factor contributing to a decrease in AEA formation might be the stress response activated by acute ethanol exposure [44]. Since ethanol can markedly increase the activity of the hypothalamus–pituitary–adrenal axis, the rise in corticosterone might affect eCB production. However, and based on the fact that restraint, a severe form of experimental stress in rats, only reduced AEA formation in the amygdala, but not in other areas such as the nucleus accumbens and cerebellum, the potential contribution of ethanol-induced stress to the inhibition of acylethanolamide release may be of less importance than that originating from ethanol-induced inhibition of both glutamate and acetylcholine release. Indeed, this experimental hypothesis needs to be confirmed.

Finally, the effects observed were tissue-selective, indicating the existence of a complex network of signals regulating eCB production. The fact that the dorsal hippocampal area is more sensitive to the inhibitory effects of ethanol on AEA and PEA formation may be dependent on the coexistence of a very important glutamatergic and cholinergic innervation, two neurotransmitters that co-operatively stimulate AEA release [40]. Indeed, all of these mechanisms may interact together in a tissue-selective fashion, in agreement with the proposed role of acylethanolamides as local mediators. The local specificity of mechanisms regulating AEA formation is supported by the activator role of ethanol in the small intestine. We have recently reported that the small intestine produces large amounts of AEA in starved animals [45]. The effects of acute ethanol on AEA production resemble those found after 18 h of food deprivation, probably because ethanol may act as a functional hypoglycaemic drug through its interference with glucose metabolism [46].

All of the effects on AEA and PEA formation in the brain described in the present paper were observed while substantial amounts of ethanol remained unmetabolized, as reflected in its plasma levels (Figure 1A). Its rapid disappearance suggests a tight regulation of acylethanolamide production, again in agreement with its role as a local mediator of intercellular signaling. Inhibition of AEA formation is regulated by rapid homoeostatic counterregulatory responses that, after repeated exposure to ethanol, led to activation of NAT, enhanced production of NAPe (AEA and PEA precursors) and increased tissue release of AEA [2,8,9].

In summary, acute exposure to ethanol inhibits acylethanolamide production in the brain through mechanisms that do not involve FAAH activity, but may be linked to neurotransmitter release. The relevance of this finding for the acute effects of ethanol, and for the neuroadaptions associated with its chronic administration, will be investigated further.

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REFERENCES

1 Hungund, B. L. and Basavarajappa, B. S. (2000) Are anandamide and cannabinoid receptors involved in ethanol tolerance?. A review of the evidence. Alcohol Alcohol. 35, 126–133


A Figure 6 Acute ethanol decreases the release of AEA and glutamate in the nucleus accumbens
(A) Time course of the effects produced by acute ethanol administration on nucleus accumbens (NAC) microdialysate AEA levels. Administration of 0.75 g of ethanol/kg of body mass (n = 6) induced a slight, but non-significant, decrease in dialysate AEA content, whereas 2 g/kg ethanol (n = 5) induced a significant reduction in dialysate AEA that persisted for at least 120 min following ethanol (EtOH) administration. Results are means ± S.E.M. of the percentage of pre-ethanol baseline AEA concentrations. *P < 0.05 as determined by post-hoc analyses following ANOVA (see text for details). (B) Time course of the effects produced by acute ethanol (EtOH) administration on nucleus accumbens microdialysate glutamate levels. Administration of 2 g of ethanol/kg of body mass (n = 5) induced a significant decrease in dialysate glutamate (GLU) levels. Results are means ± S.E.M. of the percentage of pre-ethanol baseline glutamate concentrations. *P < 0.05 as determined by post-hoc analyses following ANOVA (see text for details).


