Regulation of brain anandamide by acute administration of ethanol

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The endogenous cannabinoid acylethanolamide AEA (arachidonoylethanolamide; also known as anandamide) participates in the neuroadaptations associated with chronic ethanol exposure. However, no studies have described the acute actions of ethanol on AEA production and degradation. In the present study, we investigated the time course of the effects of the intraperitoneal administration of ethanol (4 g/kg of body mass) on the endogenous levels of AEA in central and peripheral tissues. Acute ethanol administration decreased AEA in the cerebellum, the hippocampus and the nucleus accumbens of the ventral striatum, as well as in plasma and adipose tissue. Parallel decreases of a second acylethanolamide, PEA (palmitoylethanolamide), were observed in the brain. Effects were observed 45–90 min after ethanol administration. *In vivo* studies revealed that AEA decreases were associated with a remarkable inhibition of the release of both anandamide and glutamate in the nucleus accumbens. There were no changes in the expression and enzymatic activity of the main enzyme that degrades AEA, the fatty acid amidohydrolase. Acute ethanol administration did not change either the activity of N-acyltransferase, the enzyme that catalyses the synthesis of the AEA precursor, or the expression of NAPE-PLD (*N*-acylphosphatidylethanolamine-hydrolysing phospholipase D), the enzyme that releases AEA from membrane phospholipid precursors. These results suggest that receptor-mediated release of acylethanolamide is inhibited by the acute administration of ethanol, and that this effect is not derived from increased fatty acid ethanolamide degradation.

Key words: alcohol, anandamide, arachidonoylethanolamide (AEA), cannabinoid, fatty acid amidohydrolase (FAAH), hippocampus.

INTRODUCTION

Several research lines have established a role for both the eCB (endocannabinoid) AEA (arachidonoylethanolamide or anandamide) and the CB_1 (cannabinoid 1) receptor in ethanol-dependence [1–3]. While administration of CB_1 receptor agonists promotes ethanol intake [4], the administration of a CB_1 receptor antagonist decreases ethanol self-administration [5], especially in animals with a history of ethanol-dependence [6] or in alcohol-preferring rat lines [7]. Following these findings, CB_1-receptor-knockout mice were found to display decreased ethanol preference, a phenotype that resembled that of wild-type littermates treated with a CB_1 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 down-regulated CB_1 receptors in the brain [10]. Finally, a gene-screening study has identified the CB_1 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 CB_1 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) formation in the brain. We selected three different brain areas characterized by the sensitivity to ethanol effects: the nucleus accumbens, where intrinsic neurons and glutamate release are inhibited by ethanol [16,17]; the dorsal hippocampus, where ethanol inhibits acetylcholine release [18], as well as glutamate release and glutamate-dependent depolarization [19–21]; and the cerebellum, where ethanol modifies the firing pattern and the glutamate response of Purkinje neurons [22]. These brain areas display relevant densities of CB_1 receptors where they participate in presynaptic control of neurotransmitter release [23–26]. We also monitored the *in vivo* and *in vitro* actions of ethanol on the enzymatic activities of NAT (N-acyltransferase) and FAAH, enzymes involved in AEA formation and degradation [15,27,28], as well as the mRNA expression of the NAPE-PLD (*N*-acylphosphatidylethanolamine-hydrolysing phospholipase D), the recently cloned enzyme that cleaves AEA from its membrane precursor [29]. The results suggest that acute administration of ethanol markedly inhibits *in vivo* formation of AEA and its related eCB PEA in the rat brain, and that this effect is not dependent on the activity of FAAH or NAT enzymes.

Abbreviations used: aCSF, artificial cerebrospinal fluid; AEA, arachidonoylethanolamide; CB_1, cannabinoid 1; eCB, endocannabinoid; FAAH, fatty acid amidohydrolase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GUS, β-glucuronidase; HB-β-CD, hydroxypropyl-β-cyclodextrin; NAPE-PLD, *N*-acylphosphatidylethanolamine-hydrolysing phospholipase D; NAT, N-acyltransferase; PEA, palmitoylethanolamide; PLD, phospholipase D.

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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 $^2H_4$-labelled AEA and PEA were synthesized as described in [30]. 1,2-Di[^{14}C]palmitoyl-*sn*-glycerophosphocholine (108 mCi/mmol) was purchased from Amersham Biosciences, and [1-^{3}H]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 anaesthetized with methoxyflurane (Schering-Plough) 0, 30 and 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 CO_2. 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), 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 anaesthetized 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 (136 mM NaCl, 6 mM KCl, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 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 CO_2. 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 CO_2. 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 CB_1 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 A_{260}/A_{280} 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): CB_1 receptor (NM_012784) forward, 5′-AGACCTCCTCTACGTGGGCTCG-3′, and reverse, 5′-GTACAGCGATGGCCAGCTGCTG-3′ (314 bp product); FAAH (U72497) forward, 5′-GTTACAGAGTGGAGA-GCTGTC-3′, and reverse, 5′-GAGGGTTACTGCAGTCAAA-GC-3′ (344 bp product); NAPE-PLD (AB112351) forward, 5′-GGAGCTTATGAGCCAAGGTG-3′, and reverse, 5′-ACTCT-CCGTGCTTCAGGATG-3′ (223 bp product); GAPDH forward, 5′-GGTGATGCTGGTGCTGAGTA-3′, and reverse, 5′-ACTGTGGTCATGAGCCCTTC-3′ (272 bp product); and GUS (β-glucuronidase) (NM_017015) as an endogenous control forward, 5′-TCCTGTACACCACCCCTACC-3′, and reverse, 5′-GCCATCCTCATCCAGAAGAC-3′ (146 bp product). Primers were obtained from Proligo.

Quantification was carried out on the basis of standard curves run simultaneously with samples [35]. CB_1 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 A_{260}, 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 $MgCl_2$ 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 (CB_1 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

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only a single product was amplified. Data for CB_1 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 45 000 ***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 [^{3}H]AEA (arachidonoyl-[1-^{3}H]ethanolamide) as a substrate, and measuring metabolized [^{3}H]AEA (as [^{3}H]ethanolamine) in the aqueous phase after chloroform extraction, as described in [34,35]. Microsomal NAT activity assays were performed using 1,2-di[^{14}C]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-naïve male Wistar rats ($n = 11$) were anaesthetized using 1–2 % isofluorane 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 % isofluorane vapour and a microdialysis probe (2 mm polyethersulfone 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 $MgCl_2$, 1.2 mM $CaCl_2$, 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 during 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 intraperitoneal 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*)-(+)-arachidonyl-2′-hydroxy-1′-propylamide] and loaded on to a pre-column (0.5 mm × 2.5 mm, Haisil HL C_{18}, 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 precolumn was reversed via a switching valve and the eCBs were delivered to a 0.3 mm × 50 mm microbore analytical column (Haisil HL C_{18} 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.

Table 1 Effects of saline on AEA levels in various brain sections

Effects of acute intraperitoneal administration of 0.9 % saline solution (1 ml//kg of body mass) on AEA levels (pmol/g of tissue) in the nucleus accumbens, cerebellum and dorsal hippocampus were measured in injection-habituated animals 0, 30, and 60 min after injection. Results are means ± S.E.M. for five to eight determinations per group. No significant differences were found between samples.

Brain section	Time after injection (min) . . .	AEA (pmol/g) 0	30	60
Nucleus accumbens		119 ± 20	98 ± 40	82 ± 29
Cerebellum		16.5 ± 1.4	14.7 ± 1.8	26.7 ± 1.9
Dorsal hippocampus		137 ± 51	134 ± 63	119 ± 41

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).

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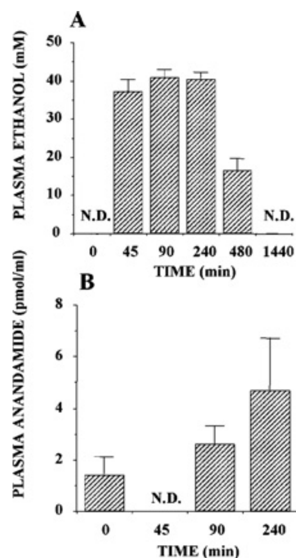

Figure 1 Time course of the effects of acute intraperitoneal administration of ethanol on plasma levels of ethanol and AEA

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.

Tissue	Time after injection (min) . . .	AEA (pmol/g) 0	45	90	240
Fat		16.2 ± 1.7	11.9 ± 0.9	19.1 ± 1.2	6.4 ± 0.6*
Liver		3.7 ± 1.8	3.7 ± 1.8	21.9 ± 3.7*	4.8 ± 2.9
Small intestine		33.9 ± 13.2	63.4 ± 23.5	285.1 ± 13.2*	62.8 ± 12.8

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).

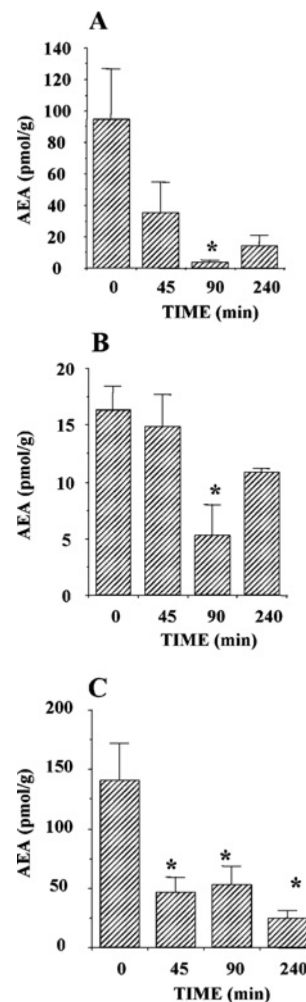

Figure 2 Time course of the effects of acute intraperitoneal administration of ethanol on tissue levels of AEA

The effects of ethanol (4 g/kg of body mass) on AEA levels 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 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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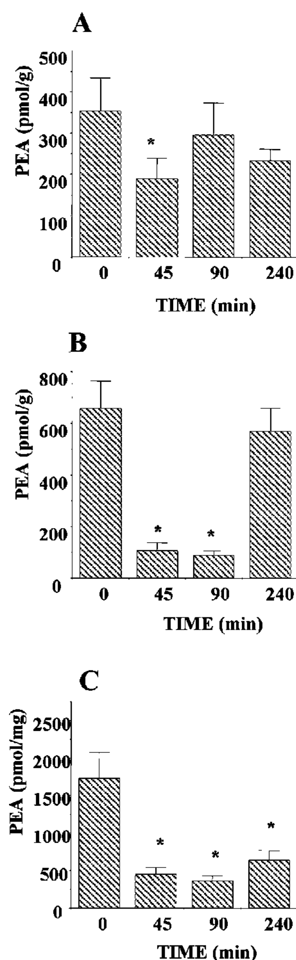

Figure 3 Time course of the effects of acute intraperitoneal administration of ethanol on tissue levels of PEA

The effects of ethanol (4 g/kg of body mass) on levels of PAE 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.

in the range of those found in plasma after the acute administration of ethanol (1 nM–10 mM; results not shown).

Acute ethanol administration does not affect the expression of CB_1 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

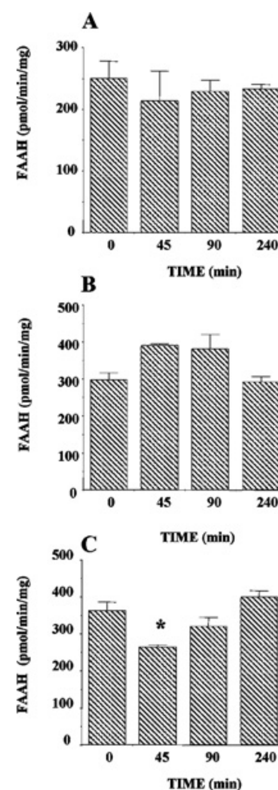

Figure 4 Time course of the effects of acute intraperitoneal administration of ethanol on the activity of FAAH in various membranes

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

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 levels. However, injection of 2 g of ethanol/kg of body mass produced a significant reduction in dialysate AEA ($F_{12,52} = 2.621$; $P < 0.001$) 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

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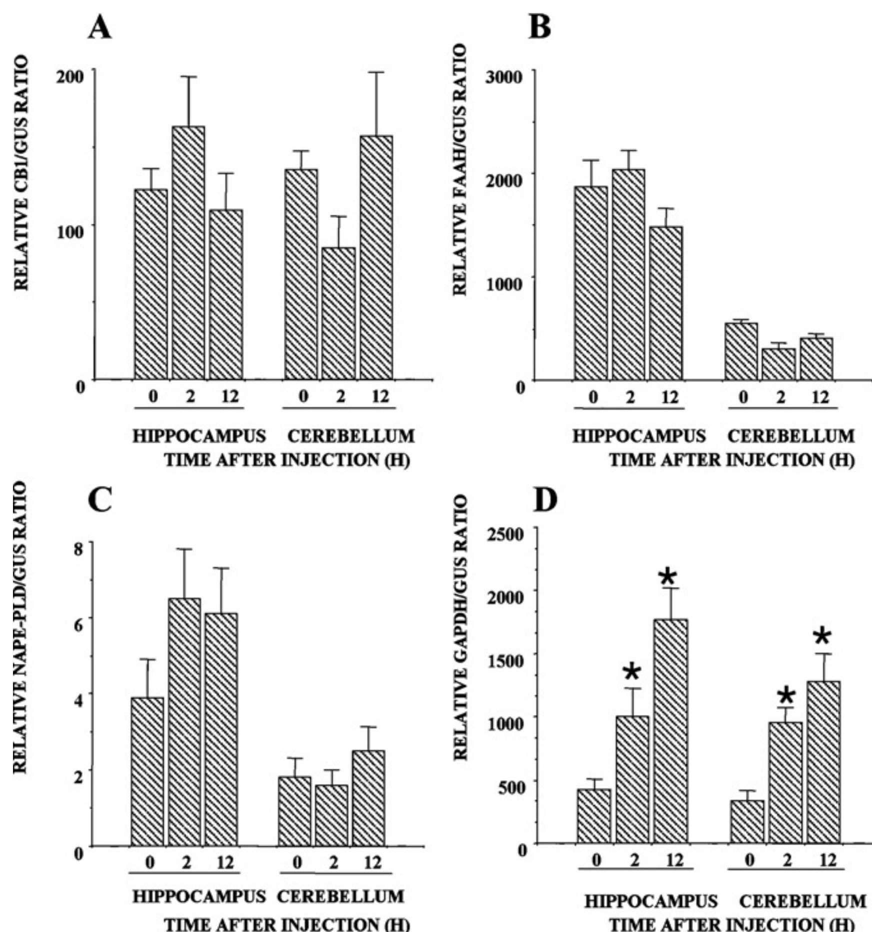

Figure 5 Time course of the effects of acute intraperitoneal administration of ethanol on CB_1 receptor, FAAH, NAPE-PLD and GAPDH mRNA expression

The effects of ethanol (4 g/kg of body mass) on mRNA expression of (**A**) CB_1 receptor, (**B**) FAAH, (**C**) NAPE-PLD and (**D**) GAPDH measured by real-time PCR in the hippocampus and cerebellum, in control animals (zero time) and alcohol-treated rats (2 and 12 h after injection). Results are means ± S.E.M. for at least seven determinations per group. $*P < 0.01$ compared with the zero-time group.

of body mass ($F_{12,82} = 4.679$; $P < 0.0001$) 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

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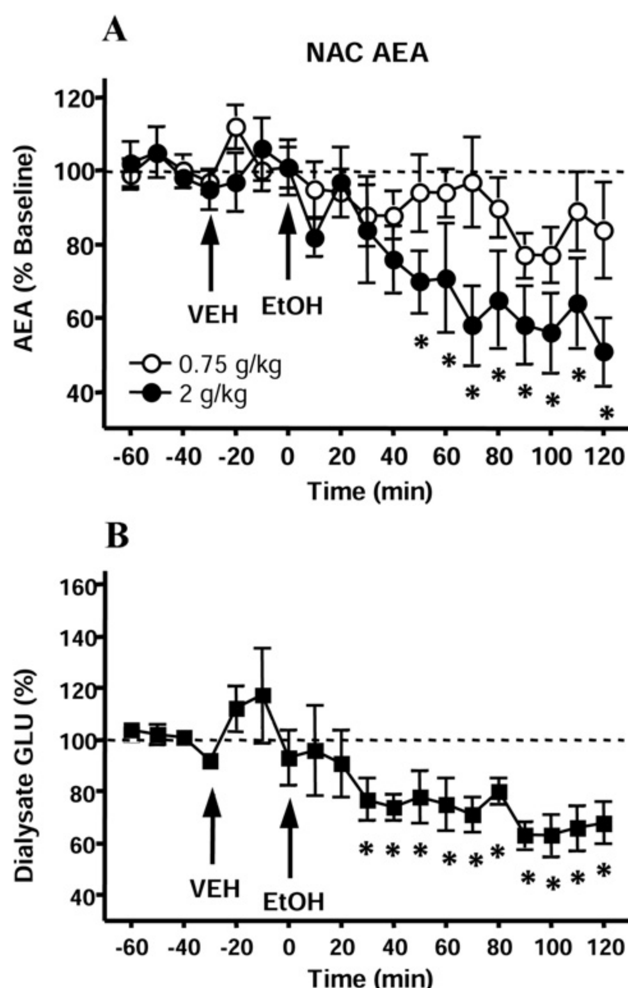

Figure 6 Acute ethanol decreases the release of AEA and glutamate in the nucleus accumbens

(**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).

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 confirms 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 hypoglycaemiant 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 NAPEs (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.

This work has been supported by Plan Nacional Sobre Drogas (F. R. de F.) and National Institute on Drug Abuse (D. P., L. H. P.) and FIS 02/001, REDES G03/05, RD06/001, MEC SAF 2004/07762 and 5th Framework Programme, grants QLRT-2000-01691 and QLRT-2001-01048 (to M. N. and F. R. de F.).

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

2 Hungund, B. L., Basavarajappa, B. S., Vadasz, C., Kunos, G., Rodríguez de Fonseca, F., Colombo, G., Serra, S., Parsons, L. and Koob, G. F. (2002) Ethanol, endocannabinoids, and the cannabinoidergic signaling system. Alcohol. Clin. Exp. Res. **26**, 565–574

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3 Wang, L., Liu, J., Harvey-White, J., Zimmer, A. and Kunos, G. (2003) Endocannabinoid signaling via cannabinoid receptor 1 is involved in ethanol preference and its age-dependent decline in mice. Proc. Natl. Acad. Sci. U.S.A. **100**, 1393–1398
4 Colombo, G., Serra, S., Brunetti, G., Gómez, R., Melis, S., Vacca, G., Carai, M. M. and Gessa, G. L. (2002) Stimulation of voluntary ethanol intake by cannabinoid receptor agonists in ethanol-preferring sP rats. Psychopharmacology **159**, 181–187
5 Arnone, M., Maruani, J., Chaperon, F., Thiebot, M. H., Poncelet, M., Soubrie, P. and Le Fur, G. (1997) Selective inhibition of sucrose and ethanol intake by SR 141716, an antagonist of central cannabinoid (CB_1) receptors. Psychopharmacology **13**, 104–106
6 Rodríguez de Fonseca, F., Roberts, A. J., Bilbao, A., Koob, G. F. and Navarro, M. (1999) Cannabinoid receptor antagonist SR141716A decreases operant ethanol self administration in rats exposed to ethanol-vapor chambers. Zhongguo Yaoli Xuebao **20**, 1109–1114
7 Cippitelli, A., Bilbao, A., Hansson, A. C., del Arco, I., Sommer, W., Heilig, M., Massi, M., Bermudez-Silva, F. J., Navarro, M, Ciccocioppo, R. and Rodríguez de Fonseca, F. (2005) Cannabinoid CB_1 receptor antagonism reduces conditioned reinstatement of ethanol-seeking behavior in rats. Eur. J. Neurosci. **21**, 2243–2251
8 Basavarajappa, B. S. and Hungund, B. L. (1999) Chronic ethanol increases the cannabinoid receptor agonist anandamide and its precursor *N*-arachidonoylphosphatidylethanolamine in SK-N-SH cells. J. Neurochem. **72**, 522–528
9 Basavarajappa, B. S., Saito, M., Cooper, T. B. and Hungund, B. L. (2000) Stimulation of cannabinoid receptor agonist 2-arachidonylglycerol by chronic ethanol and its modulation by specific neuromodulators in cerebellar granule neurons. Biochim. Biophys. Acta **1535**, 78–86
10 Basavarajappa, B. S., Cooper, T. B. and Hungund, B. L. (1998) Chronic ethanol administration down-regulates cannabinoid receptors in mouse brain synaptic plasma membrane. Brain Res. **793**, 212–218
11 Rimondini, R., Arlinde, C., Sommer, W. and Heilig, M. (2002) Long-lasting increase in voluntary ethanol consumption and transcriptional regulation in the rat brain after intermittent exposure to alcohol. FASEB J. **16**, 27–35
12 Comings, D. E., Muhleman, D., Gade, R., Johnson, P., Verde, R., Saucier, G. and MacMurray, J. (1997) Cannabinoid receptor gene (*CNR1*): association with i.v. drug use. Mol. Psychiatry **2**, 161–168
13 Schmidt, L. G., Samochowiec, J., Finckh, U., Fiszer-Piosik, E., Horodnicki, J., Wendel, B., Rommelspacher, H. and Hoehe, M. R. (2002) Association of a CB_1 cannabinoid receptor gene (*CNR1*) polymorphism with severe alcohol dependence. Drug Alcohol Depend. **65**, 221–224
14 Sipe, J. C., Chiang, K., Gerber, A. L., Beutler, E. and Cravatt, B. F. (2002) A missense mutation in human fatty acid amide hydrolase associated with problem drug use. Proc. Natl. Acad. Sci. U.S.A. **99**, 8394–8399
15 Cravatt, B. F., Giang, D. K., Mayfield, S. P., Boger, D. L., Lerner, R. A. and Gilula, N. B. (1996) Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. Nature **384**, 83–87
16 Criado, J. R., Lee, R. S., Berg, G. I. and Henriksen, S. J. (1995) Sensitivity of nucleus accumbens neurons *in vivo* to intoxicating doses of ethanol. Alcohol. Clin. Exp. Res. **19**, 164–169
17 Yan, Q., Reith, M. E. A., Yan, S. G. and Jobe, P. C. (1998) Effects of systemic ethanol on basal and stimulated glutamate releases in the nucleus accumbens of freely moving Sprague–Dawley rats: a microdialysis study. Neurosci. Lett. **258**, 29–32
18 Henn, C., Loffelholz, K. and Klein, J. (1998) Stimulatory and inhibitory effects of ethanol on hippocampal acetylcholine release. Naunyn Schmiedeberg's Arch. Pharmacol. **357**, 640–647
19 Lovinger, D. M., White, G. and Weight, F. F. (1989) Ethanol inhibits NMDA-activated ion current in hippocampal neurons. Science **243**, 1721–1724
20 Martin, D. and Swartzwelder, H. S. (1992) Ethanol inhibits release of excitatory aminoacids from slices of hippocampal area CA1. Eur. J. Pharmacol. **219**, 469–472
21 Moghadam, B. and Bolinao, M. L. (1994) Biphasic effect of ethanol on extracellular accumulation of glutamate in the hippocampus and the nucleus accumbens. Neurosci. Lett. **178**, 99–102
22 Franklin, C. L. and Gruol, D. L. (1987) Acute ethanol alters the firing pattern and glutamate response of cerebellar Purkinje neurons in culture. Brain Res. **416**, 205–218
23 Herkenham, M., Lynn, A. B., Johnson, M. R., Melvin, L. S., de Costa, B. R. and Rice, K. C. (1991) Characterization and localization of cannabinoid receptors in rat brain: a quantitative in vitro autoradiographic study. J. Neurosci. **11**, 563–583
24 Kreitzer, A. C., Carter, A. G. and Regehr, W. G. (2002) Inhibition of interneuron firing extends the spread of endocannabinoid signaling in the cerebellum. Neuron **34**, 787–796
25 Maejima, T., Hashimoto, K., Yoshida, T., Aiba, A. and Kano, M. (2001) Presynaptic inhibition caused by retrograde signal from metabotropic glutamate to cannabinoid receptors. Neuron **31**, 463–475
26 Wilson, R. I. and Nicoll, R. A. (2001) Endogenous cannabinoids mediate retrograde signalling at hippocampal synapses. Nature **410**, 588–592
27 Di Marzo, V., Fontana, A., Cadas, H., Schinelli, S., Cimino, G., Schwartz, J. C. and Piomelli, D. (1994) Formation and inactivation of endogenous cannabinoid anandamide in central neurons. Nature **372**, 686–691
28 Cadas, H., Gaillet, S., Beltramo, M., Venance, L. and Piomelli, D. (1996) Biosynthesis of an endogenous cannabinoid precursor in neurons and its control by calcium and cAMP. J. Neurosci. **16**, 3934–3942
29 Okamoto, Y., Morishita, J., Tsuboi, K., Tonai, T. and Ueda, N. (2004) Molecular characterization of a phospholipase D generating anandamide and its congeners. J. Biol. Chem. **279**, 5298–5305
30 Giuffrida, A., Rodríguez de Fonseca, F. and Piomelli, D. (2000) Quantification of bioactive acylethanolamides in rat plasma by electrospray mass spectrometry. Anal. Biochem. **280**, 87–93
31 Valenti, M., Vigano, D., Casico, M. G., Rubino, T., Steardo, L., Parolaro, D. and Di Marzo, V. (2004) Differential diurnal variations of anandamide and 2-arachidonoyl-glycerol levels in rat brain. Cell. Mol. Life Sci. **61**, 945–950
32 Patel, S., Roelke, C. T., Rademacher, D. J. and Hillard, C. J. (2005) Inhibition of restraint stress-induced neural and behavioural activation by endogenous cannabinoid signalling. Eur. J. Neurosci. **21**, 1057–1069
33 Paxinos, G. and Watson, C. (1997) The Rat Brain in Stereotaxic Coordinates, 3rd edn, Academic Press, San Diego
34 Rodríguez de Fonseca, F., Navarro, M., Gómez, R., Escuredo, L., Nava, F., Fu, J., Murillo-Rodríguez, E., Giuffrida, A., LoVerme, J., Gaetani, S. et al. (2001) An anorexic lipid mediator regulated by feeding. Nature **414**, 209–212
35 Hansson, A. C., Bermudez-Silva, F. J., Malinen, H., Hyytia, P., Sanchez-Vera, I., Rimondini, R., Rodríguez de Fonseca, F., Kunos, G., Sommer, W. H. and Heilig, M. (2007) Genetic impairment of frontocortical endocannabinoid degradation and high alcohol preference. Neuropsychopharmacology **32**, 117–126
36 Cadas, H., di Tomaso, E. and Piomelli, D. (1997) Occurrence and biosynthesis of endogenous cannabinoid precursor, *N*-arachidonoyl phosphatidylethanolamine, in rat brain. J. Neurosci. **17**, 1226–1242
37 Giuffrida, A., Parsons, L. H., Kerr, T. M., Rodríguez de Fonseca, F., Navarro, M. and Piomelli, D. (1999) Dopamine activation of endogenous cannabinoid signaling in dorsal striatum. Nat. Neurosci. **2**, 358–363
38 Walker, J. M., Huang, S. M., Strangman, N. M., Tsou, K. and Sanudo-Pena, M. C. (1999) Pain modulation by release of the endogenous cannabinoid anandamide. Proc. Natl. Acad. Sci. U.S.A. **96**, 12198–12203
39 Hansen, H. S., Lauritzen, L., Strand, A. M., Vinggaard, A. M., Frandsen, A. and Schousboe, A. (1997) Characterization of glutamate-induced formation of *N*-acylphosphatidyl-ethanolamine and *N*-acylethanolamine in cultured neocortical neurons. J. Neurochem. **69**, 753–761
40 Stella, N. and Piomelli, D. (2001) Receptor-dependent formation of endogenous cannabinoids in cortical neurons. Eur. J. Pharmacol. **425**, 189–196
41 Dahchour, A. and de Witte, P. (2000) Taurine blocks the glutamate increase in the nucleus accumbens microdialysate of ethanol-dependent rats. Pharmacol. Biochem. Behav. **65**, 345–350
42 Imperato, A., Dazzi, L., Carta, G., Colombo, G. and Biggio, G. (1998) Rapid increase in basal acetylcholine release in the hippocampus of freely moving rats induce by withdrawal from long-term ethanol intoxication. Brain Res. **784**, 347–350
43 Kötter, K. and Klein, J. (1999) Ethanol inhibits astroglial cell proliferation by disruption of phospholipase D-mediated signaling. J. Neurochem. **73**, 2517–2523
44 Rivier, C. (1993) Acute interactions between cytokines and alcohol on ACTH and corticosterone secretion in the rat. Alcohol. Clin. Exp. Res. **17**, 946–950
45 Gómez, R., Navarro, M., Ferrer, B., Trigo, J. M., Bilbao, A., del Arco, I., Cippitelli, A., Nava, F., Piomelli, D. and Rodríguez de Fonseca, F. (2002) A peripheral mechanism for CB_1 cannabinoid receptor-dependent modulation of feeding. J. Neurosci. **22**, 9612–9617
46 Williams, H. E. (1984) Alcoholic hypoglycemia and ketoacidosis. Med. Clin. North Am. **68**, 33–38

Received 20 December 2006; accepted 15 February 2007
Published as BJ Immediate Publication 15 February 2007, doi:10.1042/BJ20061898

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