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*Regulation of brain anandamide*

*by acute administration of ethanol*

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Running Title: Anandamide and acute ethanol

## ABSTRACT

The endogenous cannabinoid anandamide participates in the neuroadaptations associated with chronic ethanol exposure. However, no studies describe the acute actions of ethanol on anandamide production and degradation. In the present study we investigated the time-course of the effects of the peripheral administration of ethanol (4 gr/kg, i.p.) on the endogenous levels of anandamide in central and peripheral tissues. Acute ethanol administration decreased anandamide in the cerebellum, the hippocampus and the ventral striatum, as well as in plasma and adipose tissue. Parallel decreases of a second acylethanolamide, palmithylethanolamide were observed in the brain. Effects were observed 45-90 min after ethanol administration. *In vivo* studies revealed that anandamide decreases were associated with a remarkable inhibition of the release of both anandamide and glutamate release in the ventral striatum. There were no changes in the expression and enzymatic activity of the main enzyme that degrades anandamide, the fatty acid amidohydrolase. Acute ethanol did not change neither the activity of n-acyltransferase, the enzyme that catalyzes the synthesis of the anandamide precursor, nor the expression of NAPE-PLD, the enzyme that releases anandamide 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 of increased fatty acid ethanolamide degradation.

**Keywords:** Cannabinoid, Alcohol, Anandamide, rat, hippocampus, fatty acid amidohydrolase

Several research lines have established a role for both the endogenous cannabinoid anandamide (AEA) and the cannabinoid CB1 receptor in ethanol dependence [1 - 3]. While administration of cannabinoid CB1 receptor agonists promotes ethanol intake [4], the administration of a cannabinoid CB1 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, cannabinoid CB1 receptor knockout mice were found to display decreased ethanol preference, a phenotype that resembled that of wild-type littermates treated with a cannabinoid 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-arachidonylphosphatidylethanolamine [8]. Chronic alcohol exposure also resulted in stimulation of 2-arachidonylglycerol, a second endocannabinoid [9]. Animal studies also revealed that chronic exposure to ethanol downregulated cannabinoid CB1 receptors in the brain [10]. Finally, a recent gene screening study has identified the cannabinoid 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 endogenous cannabinoid 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 cannabinoid CB1 receptor [12, 13] or the fatty acid amidohydrolase (FAAH) [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 addressed this issue by analyzing the time-course of the effects of acute ethanol on AEA and the related mediator palmithylethanolamide (PEA) formation in the brain. We selected three different brain areas characterized by the sensitivity to ethanol effects: the ventral-striatum 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 cannabinoid CB1 receptors where they participate on presynaptic control of neurotransmitter release [23 - 26]. We also monitored the *in vivo* and *in vitro* actions of ethanol on the enzymatic activities of N-acyltransferase (NAT) and FAAH, enzymes involved in AEA formation and degradation [27, 28, 15], as well as the mRNA expression of the n-acylphosphatidylethanolamide-specific phospholipase D, the recently cloned enzyme that cleaves anandamide from its membrane precursor [29]. The results suggest that acute administration of ethanol markedly inhibits *in vivo* formation of AEA and its related endocannabinoid palmithylethanolamide in the rat brain and that this effect is not dependent on the activity of FAAH or NAT enzymes.

## MATERIAL AND METHODS

### *Animals*

Male Wistar rats (Charles River, Wilmington, MA and Barcelona, Spain), weighing  $350 \pm 50$  g were housed individually with food and water available ad libitum.

Animals were kept in an standard 12 h light/dark photoperiod (Lights on 8:00 to 20:00 a.m). They were handled daily for a week before starting the experiments, and they received i.p saline injections, once a day along the three days that preceeded 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*

Unlabeled anandamide (AEA) and palmithylethanolamide standards and [ $^2\text{H}_4$ ]-labeled anandamide palmithylethanolamide and were synthesized as described [30]. 1,2-Di[ $^{14}\text{C}$ ]palmityl-*sn*-glycerophosphocholine (108 mCi/mmol) was purchased from Amersham Bioscences (Barcelona, Spain), and [ $1\text{-}^3\text{H}$ ] ethanolamine hydrochloride (60 Ci/mmol) was from American Radiolabeled Chemicals (St. Louis, MO).

### *Vehicle and ethanol administration: Blood and tissue sampling*

Ethanol was dissolved in sterile saline (sodium chloride, 0.9% w/v) before i.p. injection. Rats received either ethanol solution (15% w/v, 4, 2 or 0.75 gr/kg) or a similar volume of a 0.9% saline solution (control group). In order to avoid potential circadian variations on endocannabinoid levels, as previously described [31] all the experiments took place between 9:00 to 13:00 a.m. during the light phase of the cycle.

Since it has been described that restraint stress may affect brain anandamide levels in specific brain areas [32], we first explore the potential effects of injection procedure on the brain levels of this endocannabinoid. To this end, we analyzed whether a single saline injection affected anandamide concentrations in the ventral striatum, cerebellum and dorsal hippocampus. A group of injection-habituated animals was anesthetized with methoxyflurane (Schering-Plough, Union, NJ) 0, 30 and 60 min after the injection of 0.9% saline (1 ml/kg). The brains were collected after rapid decapitation and immediately frozen in dry ice. Dissection of frozen brains was performed at  $-10^{\circ}\text{C}$ . Brains were placed in acrylic rat brain matrices, and 2 mm thick slices were obtained using brain matrix razor blades. The target brain region were collected using a scalpel (cerebellum and dorsal hippocampus) or a sample corer (ventral striatum). The ventral-striatum-accumbens, dorsal hippocampus and cerebellum were dissected in 2 mm-thick frozen coronal cuts, corresponding to plates 9-13 (ventral striatum-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 endocannabinoid content, two more experiments were performed. For blood and visceral sampling, a group of animals was anesthetized with methoxyfluran 0, 45, 90 and 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 (in mM: NaCl 136, KCl 5,  $\text{MgCl}_2$  1.2,  $\text{CaCl}_2$  2.5, glucose 10, Trizma base 20; pH 7.4). Blood samples were centrifuged in accuspin tubes (Sigma, St. Louis, MO) and plasma layers used for monitoring plasma AEA as described [30]. Samples were obtained from perirenal fat, liver and ileum and immediately frozen in dry ice. A second group of animals were sacrificed following the above described protocol 0, 45, 90 and 240 min after the injection of ethanol. The

brains were collected and immediately frozen in dry ice. The ventral-striatum-accumbens, dorsal hippocampus and cerebellum were dissected as described above.

#### *Plasma ethanol levels*

Plasma ethanol levels were measured enzymatically using a commercial kit (Sigma, St. Louis, MO, USA). Assays were run following the manufacturer's instructions.

#### *High-performance liquid chromatography/mass spectrometry (HPLC/MS) analyses of tissular contents of acylethanolamides*

AEA and PEA were solvent-extracted from plasma, brain areas (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, FAAH, NAPE-PLD and GAPDH mRNA expression*

Total RNA from ventral striatum-accumbens, hippocampus and cerebellum was obtained using Trizol reagent (Gibco BRL Life Technologies, Baltimore, Maryland) according to the manufacturer's instructions. All RNA samples showed A260/280 ratios between 1.8 and 2.0. First strand synthesis from each sample was done using random hexamer primer and M-MuLV reverse transcriptase (Roche Applied Science, Indianapolis, USA) according to manufacturer's instructions. Negative control reactions omitted reverse transcriptase. Resulting cDNAs were used as the template for real-time quantitative PCR, which was performed on the LightCycler instrument (Roche) with the SYBR Green I detection format.



The following primers were used (Accession numbers from NCBI database in parenthesis): CB1 (NM\_012784) forward: 5'-agacctctctacgtgggctcg-3', reverse: 5'-gtacagcgatggccagctgctg-3' (314 bp product); FAAH (U72497) forward: 5'-gttacagagtggagagctgtc-3', reverse: 5'-gagggttactgcagtcaaagc-3' (344 bp product); NAPE-PLD (AB112351) forward: 5'-ggagcttatgagccaaggtg-3', reverse: 5'-actctccgtgcttcaggatg-3' (223 bp product); GAPDH forward, 5'-ggtgatgctggtgctgagta-3', GAPDH reverse, 5'-actgtggtcatgagcccttc-3' (272 bp product). Endogenous control: beta-glucuronidase (GUS, NM\_017015) forward: 5'-tctgtacaccaccctacc-3', reverse 5'-gccatcctcatccagaagac-3' (146 bp product). Primers were obtained from Proligo (Proligo France SAS, Paris, France).

Quantification was carried out on the basis of standard curves run simultaneously than samples [35]. CB1, FAAH, NAPE-PLD, GAPDH and GUS standards were generated by PCR-amplification from control samples. The PCR product was run in a 1% agarose gel electrophoresis for checking the fragment size and the absence of other contaminant fragments, quantified by 260 nm absorbance, and serially diluted to  $10^{-5}$  pg/ $\mu$ l. Several 10-fold dilutions ( $10^{-1}$ - $10^{-5}$ ) were checked for optimal cycling on the LightCycler and three of them were selected for standard curves taking in consideration mRNA abundance. Each reaction was run in duplicate and contained 3  $\mu$ l of cDNA template, 3 mM  $Cl_2Mg$  and 0.5  $\mu$ M of primers in a final reaction volume of 20  $\mu$ l. Cycling parameters were 95°C for 10 min to activate DNA polymerase, then 30-40 cycles of 95°C for 10 s, annealing temperature for 10 s (CB1: 62°C, FAAH: 41°C, NAPE-PLD: 57°C, GAPDH: 66°C; GUS: 61°C ) and a final extension step of 72°C for 16 s in which fluorescence was acquired. Melting curves analysis were performed to ensure only a single product was amplified. Data for CB1, 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 centrifuged first at  $1000 \times g$  (5 min), the pellet was then discarded and the supernatant centrifuged at  $45,000 \times g$  (30 min). The pellets obtained were solubilized at 0-4°C in Tris buffer. The protein content in the membrane fraction was measured with 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 arachidonoyl-[1-<sup>3</sup>H]-ethanolamide as a substrate, and measuring metabolized [<sup>3</sup>H]anandamide (as [<sup>3</sup>H]ethanolamine) in the aqueous phase after chloroform extraction, as described [35, 34]. Microsomal NAT activity assays were performed using 1,2-Di[<sup>14</sup>C]palmityl-*sn*-glycerophosphocholine as substrate [36]. Both enzymatic assays were run under conditions that were linear in time and protein concentration.

*In vivo microdialysis of anandamide and glutamate*

Drug-naïve male Wistar rats (n = 11; 300-350g; Charles River, Wilmington, MA USA) were anesthetized (Isoflurane 1-2% vapor) and implanted with microdialysis guide cannula (20-gauge, SciPro Inc., Sanborn NY, USA) aimed at the nucleus accumbens shell (from Bregma: AP +1.6 mm, ML +/- 0.8 mm, and DV - 5.7 mm from dura; [33,37]). Seven days of post-operative recovery period was allowed before testing. On the day before microdialysis, animals were anesthetized (Isoflurane 1-2% vapor) and a microdialysis probe (2 mm PES membrane, 15 kDa MW cutoff, SciPro

Inc., Sanborn NY, USA) was inserted and secured to the previously implanted guide cannula. Probes were perfused overnight with artificial cerebrospinal fluid (aCSF; 145 mM NaCl, 2.8 mM KCl, 1.2 mM MgCl<sub>2</sub>, 1.2 mM CaCl<sub>2</sub>, 5.4 mM D-glucose, 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) hydroxypropyl-β-cyclodextrin (HB-β-CD) and the flowrate 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 and colleagues [38]). Following a 90 minute equilibration period dialysate samples were collected at 10-minute intervals during a 40 minute baseline period, for 30 minutes following vehicle injection (ethanol:emulphor:saline, 1:1:18; 1ml/kg) and finally for 120 minutes after ethanol administration (0.75 or 2 g/kg, i.p.; delivered as 20% (w/v)). Dialysate samples were frozen immediately following collection and stored at -80°C until analysis for eCB content using liquid chromatography coupled with mass spectrometry (LC-MS) as described below.

A second group of male Wistar rats (n = 5) were surgically implanted with microdialysis guide cannulae aimed at the NAC for the analysis of ethanol-induced alterations in interstitial glutamate (GLU) 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 faster perfusate flowrate was employed (1 µl/min) to allow sampling splitting for the monoamine and amino acid analyses (see below).

*LC-MS Analysis of dialysate endocannabinoid content*

Dialysate levels of AEA were determined using liquid chromatography coupled with ESI mass spectrometry (LC-MS). 5  $\mu$ l microdialysate aliquots were spiked with 5  $\mu$ l of 100 nM of (S)-(+)-arachidonyl-2'-hydroxy-1'-propylamide (S-2 methanandamide) and loaded onto a pre-column (0.5 x 2.5 mm, Haisil HL C18 5 $\mu$ m; Higgins Analytical Inc., Mountain View, CA) using a 30% MeOH (v/v) mobile phase delivered at 70  $\mu$ l/min. Following a 2 min wash period, mobile phase flow through the precolumn was reversed via a switching valve and the eCBs were delivered to a 0.3 x 50 mm microbore analytical column (Haisil HL C18 3 $\mu$ m; Higgins Analytical Inc., Mountain View, CA) using an isocratic mobile phase consisting of 70% MeOH (v/v) delivered at 5  $\mu$ l/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 find 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 quantitation were approximately 0.1 nM.

*Analysis of dialysate glutamate*

Microdialysis samples were split into two 5  $\mu$ l aliquots for quantification of glutamate content by capillary electrophoresis coupled with laser-induced fluorescence detection (CE-LIF). Specific details on these analyses have been previously published [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 anandamide levels*

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

### *Acute ethanol decreases plasma anandamide levels*

The acute i.p. administration of 4 gr/kg of ethanol led to a rapid rise in plasma ethanol levels, which then remained stable for 4 hr after the administration (Fig 1A).

Clearance of plasma ethanol was evident 8 hr after the injection and was complete within the first 24 hr. Plasma AEA levels were affected after acute ethanol exposure (Fig 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 hr and returned to control levels at 8 hr ( $1.3 \pm 0.6$  pmol/ml,  $n = 8$ ) and 24 hr ( $0.7 \pm 0.5$  pmol/ml,  $n = 8$ ).

In peripheral tissues, ethanol decreased AEA levels in perirenal fat, but resulted in a marked increase in the small intestine and liver 90 min after the injection (Table 2). Based on both the presence of stable ethanol levels and on the changes in plasma AEA, we selected the time points 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 decreases anandamide and palmithylethanolamide in the ventral striatum, cerebellum and hippocampus*

Acute injection of ethanol decreased AEA levels in the ventral striatum (Fig 2A), cerebellum (Fig 2B) and hippocampus (Fig 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 ventral striatum (Fig 3A) 45 min after its injection, the cerebellum at 45 and 90 min (Fig 3B) and the hippocampus in all time points studied (Fig 3C).

*Acute ethanol does not increase FAAH activity*

In order to investigate whether the decrease on the levels of AEA and PEA observed after acute ethanol might be derived of 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 neither in membranes from ventral-striatum nor from cerebellum (Figure 4A and 4B). However, FAAH activity was decreased 45 min after alcohol administration in the hippocampus, normalizing thereafter. Additional studies performed *in vitro* studies using cerebellar membranes indicated that FAAH activity

was not affected by the presence of ethanol in the buffer at concentrations in the range of those found in plasma after the acute administration of ethanol ( $10^{-9}$  to  $10^{-2}$  M, data not shown).

*Acute ethanol does not affect the expression of CB<sub>1</sub> FAAH and NAPE-PLD mRNA in the hippocampus and 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 the n-acylphosphatidyl-ethanolamide-specific phospholipase D, the recently cloned enzyme that cleaves anandamide from its membrane precursor (Fig 5A to 5D). Any of the endocannabinoid-related genes were affected by acute ethanol administration either at 2 or 12 hr after the injection. However, GADPH expression was clearly increased after acute alcohol exposure. This pattern was observed also both in the hippocampus and in the cerebellum. These results point against an activation of degradation as the main origin of the ethanol-induced decrease of anandamide in the brain.

*Acute ethanol does not affect NAT activity*

The activity of the enzyme involved in the synthesis of the AEA precursor N-arachidonoyl phosphatidyl ethanolamine - NAT - was not affected in cerebellar membranes after acute i.p. administration of ethanol (Table 3). We could not measure NAT activity on individual hippocampus because the technique may require pooling of various samples in order to reach sufficient amount of protein.

*Acute ethanol decreases anandamide in the ventral striatum*

Baseline dialysate AEA concentrations were  $2.03 \pm 0.5$  and  $2.12 \pm 1.1$  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 ( $F(1,141) = 25.094$ ,  $p < 0.001$ ; see Figure 6A). Administration of 0.75 g/kg ethanol produced a subtle and non-significant decrease in dialysate AEA levels. However, injection of 2 g/kg ethanol produced a significant reduction in dialysate AEA ( $F_{12,52} = 2.621$ ;  $p < 0.001$ ) which was evident within 40 minutes after administration and persisted at approximately 60% of pre-ethanol baseline levels for the remainder of the 120 minute post-ethanol sample collection period.

*Acute ethanol decreases glutamate in the ventral striatum*

Baseline dialysate glutamate levels in the same samples were  $602 \pm 21$  nM. Glutamate levels in the dialysate samples were significantly reduced following 2 g/kg ethanol ( $F_{12,82} = 4.679$ ;  $p < 0.0001$ ) which was evident within 30 minutes after administration and persisted at approximately 70% of pre-ethanol baseline levels for the remainder of the 120 minute 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 (Fig 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 endogenous cannabinoid targets for ethanol. Increases in AEA levels in tissues may be dependent either on changes in the synthesis of AEA precursor n-arachydonylphosphatidylethanolamide (NAPE), or on the cleavage of NAPEs species by receptor-activated phospholipase D (PLD), or by blockade of FAAH-dependent enzymatic degradation of AEA [28, 15, 27]. As there was no activation of FAAH by *in vivo* or *in vitro* ethanol, and since initial observations indicate 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 might operate then through the

modulation of neurotransmitters capable of activate specific receptors coupled to NAPE-PLD. This mechanism may not request a substantial modification of NAPE-PLD production because the inhibition of the release of neurotransmitters will solely result in reduced endocannabinoid production. Following this rationale, we observed that the expression of this synthetic enzyme is not affected 2 hr 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 endocannabinoid 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 confirms this hypothesis, since we found parallel decreases in extracellular levels of both, AEA and glutamate, in the ventral striatum (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]. Second, ethanol may directly inhibit glutamate NMDA 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 signaling, acting as a metabolic substrate that shifts PLD activity to the production of the phospholipid phosphatidylethanol [43], this hypothesis can not 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 anandamide 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 endocannabinoid production. However, and based on the fact that restraint, a severe form of experimental stress in rats, only reduced anandamide 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 acylethanolamides release may be of less importance than that originated by 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 endocannabinoid 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 cooperatively stimulate AEA release [40]. Indeed, all these mechanisms may interact together in a tissue-selective fashion, in agreement with the proposed role for 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 hypoglycemic drug through its interference with glucose metabolism [46].

All the effects on AEA and PEA formation in the brain described in the present study were observed while substantial amounts of ethanol remained unmetabolized, as reflected in its plasma levels (Fig 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 anandamide formation is regulated by rapid homeostatic counterregulatory responses that, after repeated exposure to ethanol, lead to an activation of NAT, an enhanced production of NAPEs (AEA and PEA precursors) and an increased tissular release of AEA [8, 9, 2].

In summary, acute exposure to ethanol inhibits acylethanolamide production in the brain through mechanisms that do not involve fatty acid amidohydrolase 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 further investigated.

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TABLE 1

Effects of acute administration of vehicle (0.9% saline solution, injected 1ml/kg i.p.) on anandamide levels (pmol/g tissue) in the ventral striatum (nucleus accumbens), cerebellum and dorsal hippocampus, measured in injection-habituated animals 0, 30, and 60 min after the injection. Data are the means  $\pm$  SEM of 5-8 determinations per group. (\*)  $P < 0.01$  versus time 0 group, Newman-Keuls.

<i>Time after the injection (min)</i>	<i>Anandamide (pmol/g)</i>		
	0'	30'	60'
<i>Ventral Striatum</i>	119 $\pm$ 20	98 $\pm$ 40	82 $\pm$ 29
<i>Cerebellum</i>	16.5 $\pm$ 1.4	14.7 $\pm$ 1.8	26.7 $\pm$ 1.9
<i>Dorsal Hippocampus</i>	137 $\pm$ 51	134 $\pm$ 63	119 $\pm$ 41

TABLE 2

Effects of acute administration of ethanol (4 gr/kg i.p.) on anandamide (AEA) levels (pmol/g tissue) in the perirenal fat, liver and small intestine, 0, 45, 90 and 240 min after the injection. Data are the means  $\pm$  SEM of 5-8 determinations per group. (\*)  $P < 0.01$  *versus* time 0 group, Newman-Keuls.

	0'	45'	90'	240'
<i>Fat</i>	16.2 $\pm$ 1.7	11.9 $\pm$ 0.9	19.1 $\pm$ 1.2	6.4 $\pm$ 0.6 *
<i>Liver</i>	3.7 $\pm$ 1.8	3.7 $\pm$ 1.8	21.9 $\pm$ 3.7 *	4.8 $\pm$ 2.9
<i>Small intestine</i>	33.9 $\pm$ 13.2	63.4 $\pm$ 23.5	285.1 $\pm$ 13.2 *	62.8 $\pm$ 12.8

TABLE 3

Lack of effect of acute administration of ethanol (4 gr/kg i.p.) on n-acyl transferase (NAT) activity in cerebellar membrane fractions, 0, 45, 90 and 240 min after the injection. Data are the means  $\pm$  SEM of 5-8 determinations per group.

	<b>0'</b>	<b>45'</b>	<b>90'</b>	<b>120'</b>
NAT (pmol/mg/min)	452 $\pm$ 108	449 $\pm$ 104	687 $\pm$ 234	385 $\pm$ 122

**FIGURE LEGENDS**

## FIGURE 1

Time-course of the effects of the acute i.p. administration of ethanol (4 gr/kg) on **A.** plasma levels of ethanol, **B.** plasma levels of anandamide. Data are the means  $\pm$  SEM of at least 5 determinations per group. (\*)  $P < 0.01$ , *versus* time 0 group, Newman-Keuls.

## FIGURE 2

Time-course of the effects of the acute i.p. administration of ethanol (4 gr/kg) on tissular levels of anandamide (AEA) in **A.** the ventral striatum-nucleus accumbens, **B.** the cerebellum and **C.** the dorsal hippocampus. Data are the means  $\pm$  SEM of at least 5 determinations per group. (\*)  $P < 0.01$ , *versus* time 0 group, Newman-Keuls.

## FIGURE 3

Time-course of the effects of the acute i.p. administration of ethanol (4 gr/kg) on tissular levels of palmithylethanolamide (PEA) in **A.** the ventral striatum-nucleus accumbens, **B.** the cerebellum and **C.** the dorsal hippocampus. Data are the means  $\pm$  SEM of at least 5 determinations per group. (\*)  $P < 0.01$ , *versus* time 0 group, Newman-Keuls.

## FIGURE 4

Time-course of the effects of the acute i.p. administration of ethanol (4 gr/kg) on the activity of fatty acid amidohydrolase in membranes from **A.** the ventral striatum-nucleus accumbens, **B.** the cerebellum and **C.** the dorsal hippocampus. Data are the



means  $\pm$  SEM of at least 7 determinations per group. (\*)  $P < 0.01$ , *versus* time 0 group, Newman-Keuls.

#### FIGURE 5

Effects of the acute i.p. administration of ethanol (4 gr/kg) on the mRNA expression of **A.** The cannabinoid CB1 receptor, **B.** the fatty acid amidohydrolase (FAAH), **C.** The n-acylphosphatidyl-ethanolamide-specific phospholipase D (NAPE-PLD) and **D.** The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) measured by real-time PCR in the hippocampus and cerebellum, in control animals (time = 0) and alcohol-treated rats (time = 2 and 12 hr after injection). Data are the means  $\pm$  SEM of at least 7 determinations per group. (\*)  $P < 0.01$ , *versus* time 0 group, Newman-Keuls.

#### FIGURE 6

Acute ethanol decreases the release of anandamide and glutamate in the ventral striatum. **A.** Time-course of the effects produced by acute ethanol administration on ventral striatum microdialysate AEA levels. Administration of 0.75 g/kg ethanol ( $n = 6$ ) 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 minutes following ethanol administration. Data are mean  $\pm$  SEM 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 administration on ventral striatum microdialysate glutamate levels. Administration of 2 g/kg ethanol ( $n = 5$ ) induced a significant decrease in dialysate glutamate levels. Data are mean  $\pm$  SEM of the percentage of pre-

ethanol baseline glutamate concentrations, respectively. \*  $P < 0.05$  as determined by post hoc analyses following ANOVA (see text for details).

FIGURE 1

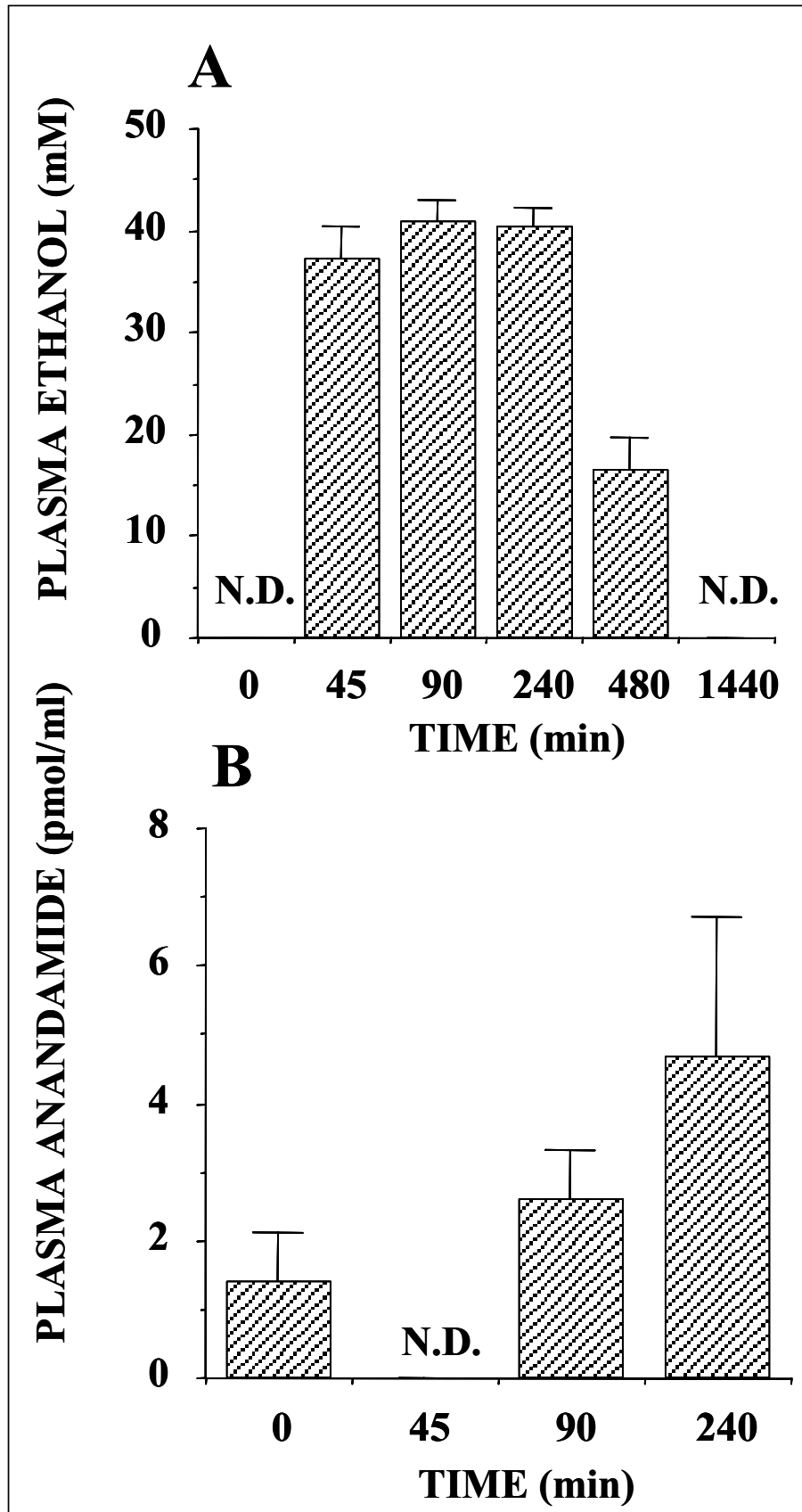
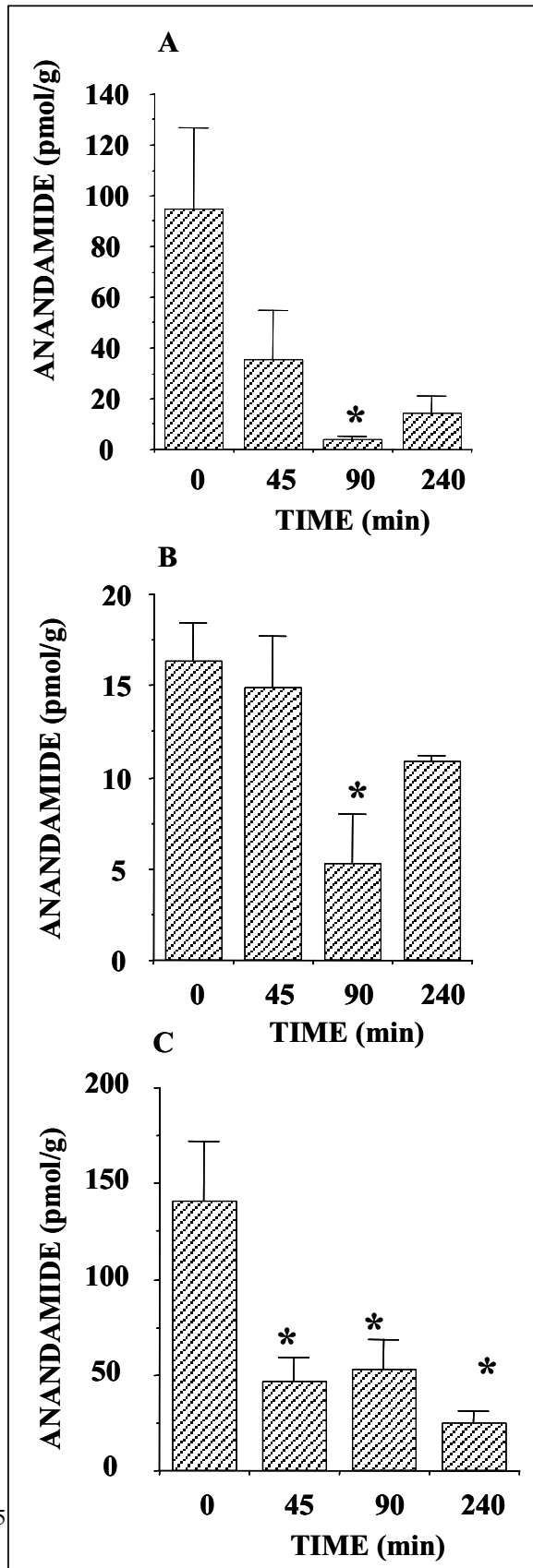


FIGURE 2



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FIGURE 3

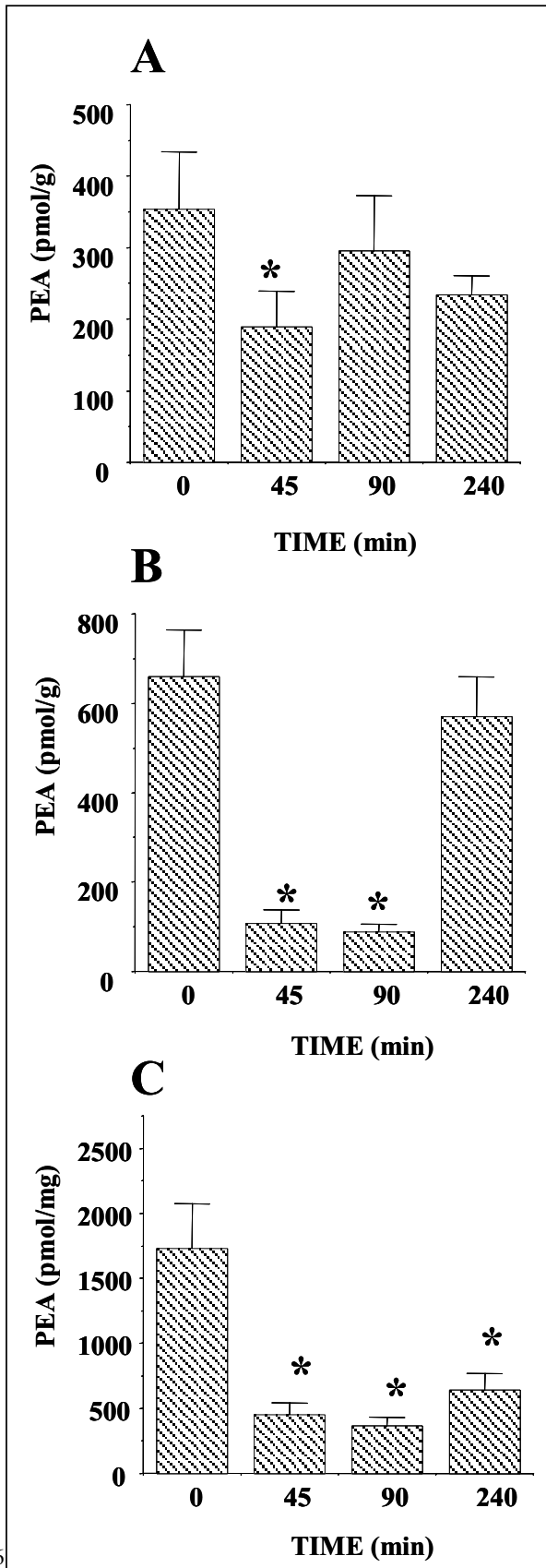


FIGURE 4

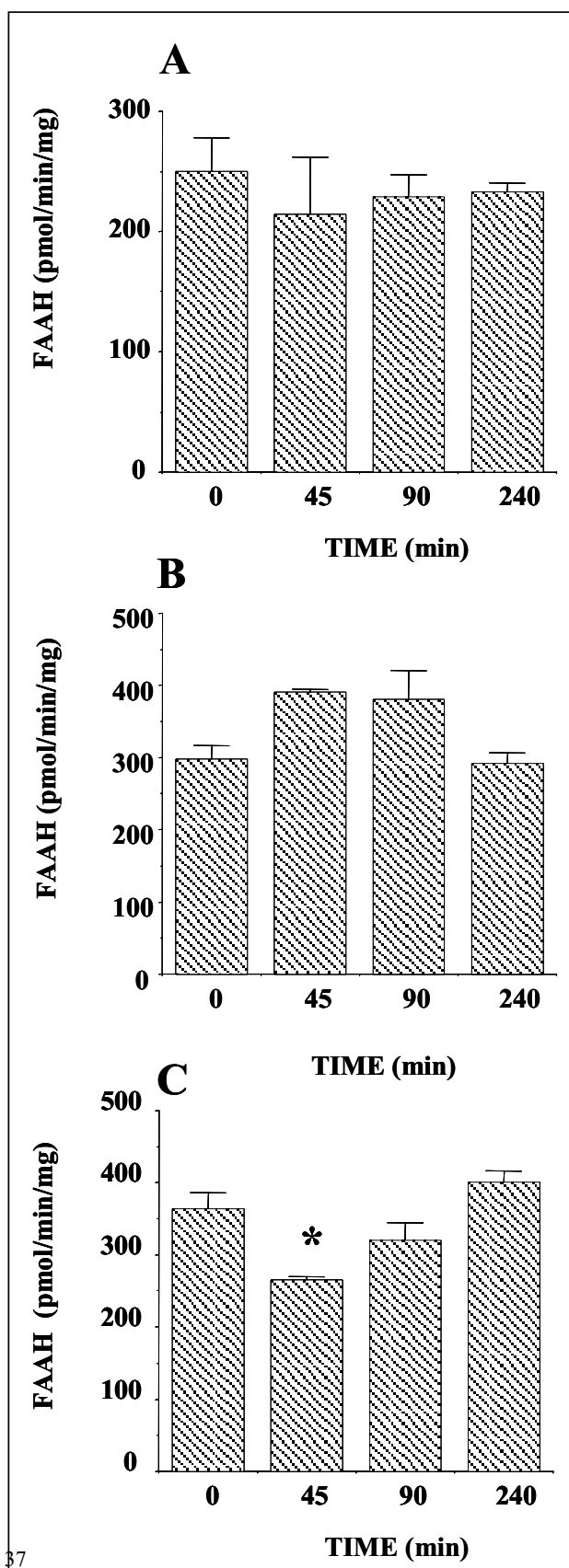


FIGURE 5

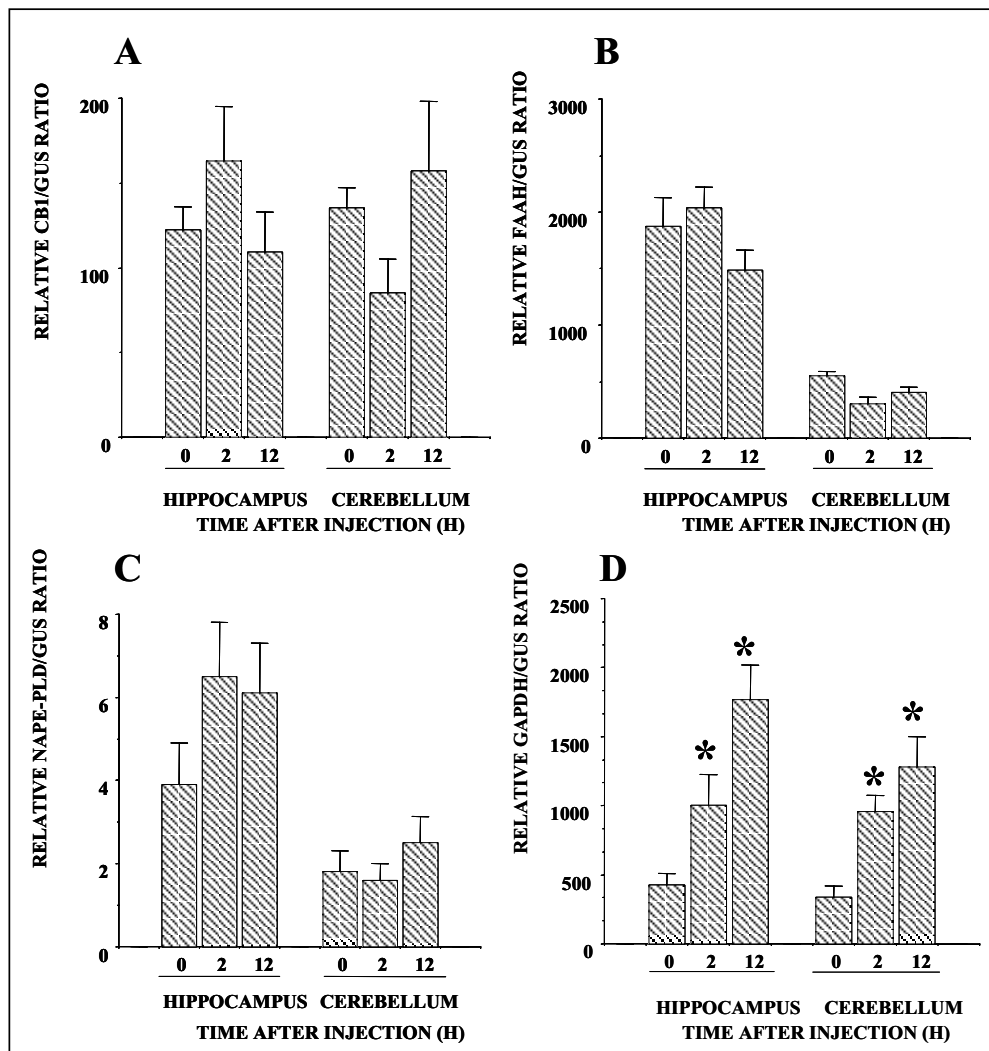


FIGURE 6

