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Ethanol downregulates *N*-acyl phosphatidylethanolamine-phospholipase D expression in BV2 microglial cells via epigenetic mechanisms

Fernando Correa *, Andrea De Laurentiis, Ana María Franchi

Center for Pharmacological and Botanical Studies, National Research Council, School of Medicine, University of Buenos Aires, Argentina

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ABSTRACT

Excessive ethanol drinking has deleterious effects on the brain. However, the effects of alcohol on microglia, the main mediator of the brain's innate immune response remain poorly understood. On the other hand, the endocannabinoid system plays a fundamental role in regulating microglial reactivity and function. Here we studied the effects of acute ethanol exposure to murine BV2 microglial cells on *N*-acyl phosphatidylethanolamine-phospholipase D (NAPE-PLD), a major synthesizing enzyme of anandamide and other *N*-acylethanolamines. We found that ethanol downregulated microglial NAPE-PLD expression by activating cAMP/PKA and ERK1/2. These signaling pathways converged on increased phosphorylation of CREB. Moreover, ethanol induced and increase in histone acetyltransferase activity which led to higher levels of acetylation of histone H3. Taken together, our results suggest that ethanol actions on microglial NAPE-PLD expression might involve epigenetic mechanisms.

1. Introduction

Alcohol abuse is one of the major public health, social and medical concerns worldwide. Despite the high prevalence of alcohol use disorder, the molecular mechanisms involved in the neuroinflammation and neurodegeneration observed in alcoholics remain poorly understood. Similarly, acute ethanol exposure has been associated with increased risk of brain damage. Alcohol intoxication increases the risk of hemorrhagic strokes (Juvela et al., 1995) and aggravates the brain injury caused by intracerebral hemorrhage (Liew et al., 2016). Likewise, ethanol exacerbates neuroinflammation caused by traumatic brain injury and interferes with neurological recovery (Teng et al., 2015). In experimental animals, excessive ethanol administration induces the expression of inflammatory mediators in the brain which are associated with brain damage (Alfonso-Loeches et al., 2010; Pascual et al., 2015; Qin et al., 2008; Vallés et al., 2004). Interestingly, it has been reported that ethanol modulates the expression of pro-inflammatory mediators via activation of toll-like receptor-4 (TLR4) since TLR4$^{-/-}$ mice are protected from ethanol-induced brain damage (Alfonso-Loeches et al., 2010; Pascual et al., 2011). Recent evidence has shown an increased microglial expression of monocyte chemoattractant protein-1 (MCP-1) and ionized binding adaptor protein-1 (Iba-1) in the brains of chronic excessive alcohol users (He and Crews, 2008). Moreover, binge alcohol drinking induces long-lasting morphological and phenotypical changes in microglia (McClain et al., 2011). However, the molecular mechanisms by which ethanol affect microglial activation and function are still poorly understood.

Microglia, the central nervous system (CNS) resident innate immune cell is considered to be the main source of cytokines and inflammatory mediators involved in neuroinflammatory processes during brain injury and neurodegeneration (reviewed by Hanisch and Kettenmann (2007)). Once microglial cells detect injury or are stimulated by an external signal, they became activated which include morphological transformation, the production of cytokines as well as other mediators and changes in the membrane receptors (Hanisch and Kettenmann, 2007). Endocannabinoids are one group of lipid mediators produced by microglial cells upon activation. In fact, cultured microglia produces approximately 20-fold more endocannabinoids than neurons and astrocytes in culture (Walter et al., 2003). Cumulative evidence shows that microglia-produced endocannabinoids act autocrinally to modulate microglial activation by changing the cytokine production pattern from pro-inflammatory to anti-inflammatory (Correa et al., 2011; Eljaschewitsch et al., 2006; Kreutz et al., 2009; Mecha et al., 2015). *N*-acylphosphatidylethanolamine-phospholipase D (NAPE-PLD) is the main synthesizing enzyme of anandamide, a major endocannabinoid, and other *N*-acylethanolamides. Interestingly, it has been reported that NAPE-PLD$^{-/-}$ mice show a defective

* Corresponding author.
E-mail address: fcorrea@fmed.uba.ar (F. Correa).

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response to pro-inflammatory stimuli (Zhu et al., 2011).

The interaction between ethanol and the endocannabinoid system (eCS) is rather complex. It has been reported that acute ethanol exposure modulates the expression of the eCS in hippocampus, nucleus accumbens, striatum, amygdala, prefrontal cortex and cerebellum (Ferrer et al., 2007; Rubio et al., 2007) producing a general reduction in the activity of this signaling system (Rubio et al., 2009). However, data regarding whether ethanol modulates the expression of the eCS in microglia is still lacking. We hypothesize that acute ethanol exposure to microglial cells modify some of the components of the eCS, thus contributing to altering microglial function. Here, we explore for the first time the effects of acute ethanol exposure on murine BV2 microglial cells NAPE-PLD expression and the molecular mechanisms involved.

2. Material and methods

2.1. Reagents

RPMI culture media and PD98059 were purchased from Invitrogen Argentina S.A. (Buenos Aires, Argentina). Foetal bovine serum (FBS) was from Natocor (Córdoba, Argentina). H-89 was supplied from Tocris Bioscience (Briston, United Kingdom). Anti-phospho-ERK1/2 and anti-phospho-CREB were from Santa Cruz (Dallas, TX, USA). Anti-acetyl-H3 was from Merck Millipore (Darmstadt, Germany). Anti-β-actin antibody, isobutylmethyl xanthine (IBMX) and cyclic adenosine monophosphate (cAMP) were from Sigma-Aldrich Co (St. Louis, USA). NAPE-PLD primary antibody was purchased from Abcam Inc. (Cambridge, MA, USA). Horse radish peroxidase conjugated anti-mouse IgG and horse radish peroxidase conjugated anti-rabbit IgG were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). Western blotting reagents and nitrocellulose membranes (Trans-Blot, 0.45 μm) were from Bio-Rad Inc. (Hercules, CA, USA) and molecular weight marker was purchased by GE Healthcare Bio-Science Corp. (Piscataway, NJ, USA). Trizol reagent, dNTPs and ribonuclease inhibitor were from Genbiotech (Buenos Aires, Argentina). Ultrapure water, DTT, RNAse free DNAse I, Moloney Murine Leukemia virus reverse transcriptase (MMLV-RT) and random primers were purchased from Invitrogen (Carlsbad, CA, USA). GoTaq DNA polymerase was provided by Promega (Madison, USA). EvaGreen® dye was from Biotium, Inc. (Hayward, CA, USA).

2.2. Cell line cultures

The murine microglial cell line BV2 was a kind gift of Dr. Keith W. Kelly (University of Illinois at Urbana-Champaign). Cells were grown in RPMI supplemented 10% FBS and 1% penicillin/streptomycin and passaged every 5 days for a maximum of 30 passages. Cells were grown in a humidified environment containing 5% CO_2 and held at a constant temperature of 37 °C. These cells exhibit morphological and functional properties comparable to primary microglia. The day before to each experiment, BV2 cells were plated in a 12-well plate at a concentration of 5.10^5 cells/ml; except for the cAMP assay, in which case cells were plated in Petri dishes at a concentration of 1.10^6 cells/ml.

2.3. Cell treatment

Prior to each experiment, medium containing serum was removed and replaced with fresh serum-free RPMI. BV2 cells were stimulated with ethanol 100 mM. The concentration of ethanol used in our in vitro experiments is not only widely used in the literature (Boyadjieva and Sarkar, 2013; Fernandez-Lizarbe et al., 2009; Lee et al., 2004; Rubio et al., 2011) but also in the range of the in vivo blood alcohol levels achieved during acute intoxications (Dolganiuc and Szabo, 2009). Cells were harvested at 24 h or 48 h post-ethanol exposure for mRNA isolation. Protein analyses for CREB and ERK1/2 phosphorylation were performed at 5, 15, 30, 45 or 60 min post-ethanol exposure. Protein analyses for H3 acetylation in response to ethanol exposure was performed a 6, 12 and 24 h (Aroor et al., 2010; Kim and Shukla, 2006; Park et al., 2003; Qiang et al., 2011).

Pharmacological inhibition of ERK1/2 signaling pathway (with PD98035 10 μM) or PKA signaling pathway (with H-89 10 μM), was performed adding the respective inhibitors 30 min before exposure to ethanol. Cells were harvested for protein or mRNA analysis 24 h post-ethanol exposure. The concentrations of PD98035 and H-89 were chosen according to the literature.

2.4. cAMP assay

BV2 cells were preincubated for 5 min IBMX 800 μM at 37 °C (t=0) followed by incubation for 5, 15, 30, 45 or 60 min with ethanol 100 mM. At the appropriate time, media were discarded and BV2 cells were washed with ice-cold PBS. Next, cells were harvested and 5 ml of ice-cold ethanol was added to preserve the intracellular cAMP levels and stored at −80 °C until further use. The day of the assay, tubes were centrifuged at 1000 g for 10 min at 4 °C. The ethanolic supernatant was separated from the precipitated protein pellet. The ethanol was evaporated from the supernatants and residues were resuspended in 50 mM Tris–HCl, pH 7.4, 0.1% BSA for cAMP determination. Cyclic AMP levels were measured by competitive radio-binding assay to the regulatory subunit of PKA using [^{3}H]cAMP, as previously described (Davio et al., 1995). Precipitated proteins were solubilized from the pellet with boiling 2% SDS for 10 min. Protein concentration was determined by the Lowry assay. The relative cAMP levels were normalized to the correspondent protein (cAMP/mg protein) and results are expressed as fold of change of control.

2.5. Western blot analysis

After treatment, cells were harvested with lysis buffer (10 mM Hepes, 5 mM $MgCl_2$, 142.5 mM KCl, 0.1% SDS, 1% Nonidet-40, 5 mM EDTA, 0.5% sodium deoxycholate in phosphate buffered saline) with a freshly added protease inhibitor cocktail (10 μg/ml leupeptin, 2 μg/ml aprotinin, 100 μg/ml soybean-trypsin inhibitor, 1 mmol/l EDTA, 1 mg/ml benzamidine, 10 μg/ml DTT and 1 mg/ml caproic acid). Cells were sonicated (Ultrasonic Cell Disrupter, Microson, Heat systems Inc.) for 30 s, centrifuged at 1000 g for 10 min and protein concentration determined by the Bradford assay. Thirty micrograms of protein were loaded in each lane and samples were separated by electrophoresis in 7.5–12% SDS-PAGE gel and transferred to a 0.45 μm nitrocellulose membrane. Membranes were blocked using 5% w/v dried non-fat milk and then incubated with the respective primary antibodies. After incubation with the primary antibody, membranes were washed with PBS-T (10 mM Tris, 100 mM NaCl and 0.1% Tween 20, pH 7.5) followed by 1 h incubation with horse radish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody and developed using the enhanced chemiluminescence system. The images for immunoreactive bands were acquired using the ImageQuant blot documentation instrument and analyzed using the Image J software package. The relative protein level was normalized to β-actin and results are expressed as relative optical density (NAPE-PLD/β-actin; pERK1/2/β-actin; pCREB/β-actin; acetyl-H3/β-actin).

2.6. RT-PCR

Cells were harvested at the end of each experiment in TRI

Reagent and frozen at −80 °C until used. Total RNA from cell cultures was isolated according to the manufacturer's recommendations (Molecular Research Center Inc., Cincinnati, OH, USA). Following extraction, RNA was quantified and cDNA was synthesized from total RNA (3 μg) using M-MLVRT, random primers and ribonuclease inhibitor. The specific primers to performed PCR amplifications were designed using the Primer3 Software. The primers sequences are: β-actin forward 5′ TGT TAC CAA CTG GGA CGA CA 3′ and reverse 5′ TCT CAG CTG TGG TGG TGA AG 3′ (94 °C 5 min, 30 cycles of: 94 °C 40 s, 57 °C 30 s, 72 °C 1 min, 72 °C 5 min); NAPE-PLD forward 5′ ATG AGA ACA GCC AGT CTC CA 3′ and reverse 5′ CCA TTT CCA CCA TCA GCG TC 3′ (94 °C 5 min, 35 cycles of: 94 °C 40 s, 57 °C 1 min, 72 °C 1 min, 72 °C 5 min). Products were loaded onto 2% agarose gel and stained with ethidium bromide. Bands were visualized on a transilluminator under UV light. Photographs were taken with a digital camera (Olympus C-5060) and analyzed with the Image J software package. The relative mRNA level was normalized to β-actin and results were expressed as relative optical density (NAPE-PLD/β-actin).

2.7. Real time PCR

Following RNA extraction, genomic DNA was digested with DNAse for 10 min a room temperature. Next, DNAsa was inactivated and the retrotranscription process was performed as previously described. Shortly, cDNA was synthesized from total RNA (3 μg) using M-MLVRT, random primers and ribonuclease inhibitor. The primers sequences are: NAPE-PLD forward 5′TAC TCA TCC ATG TCC CTC GG 3′ and reverse 5′ ATA TCT GCG TGG AAC AGC CT 3′ and GAPDH forward 5′ TCA CTG GCA TGG CCT TCC 3′ and reverse 5′ GGC GGC ACG TCA GAT CC 3′. Amplification conditions were 94 °C 5 min, 40 cycles of: 94 °C 30 s, 60 °C 30 s, 72 °C 30 s, 72 °C 5 min. Real-time PCR was followed with EvaGreen® dye. NAPE-PLD gene expression was analyzed with the ΔΔCt method for relative quantifications and normalized to GAPDH (NAPE-PLD/GAPDH).

2.8. Histone acetyltransferase assay

Histone acetyltransferase (HAT) activity assay was performed using the commercial colorimetric kit from Sigma-Aldrich Co (St. Louis, USA) and following the manufacture's recommendations. The colorimetric signal was measured in a plate spectrophotometer (Bio-Rad Inc.; Hercules, CA, USA) at 450 nm. Values are expressed as percentage of change relative to control (100%).

2.9. Statistical analysis

Results are presented as means ± S.E.M. of at least three experiments performed with different cell preparations. Analysis of variance followed by the Tukey's test for multiple comparison was used to determine statistical significance (95%; $P < 0.05$). Normality and homoscedasticity were tested by Shapiro–Wilk (modified) and Levene test, respectively. Statistical analysis was performed using the software Infostat (Córdoba, Argentina).

3. Results

3.1. Acute exposure to ethanol downregulates microglial expression of NAPE-PLD

Acute ethanol exposure to microglia has been shown to alter its phenotype and function (Bell-Temin et al., 2013). However, little is known on the effects of alcohol on the microglial endocannabinoid system. Therefore, our first approach was to study the in vitro effect of acute ethanol exposure on microglial NAPE-PLD expression (the main synthesizing enzyme of anandamide). We incubated the murine BV2 microglial cell line with ethanol (100 mM) for 24 h and proceeded to analyze the NAPE-PLD protein and mRNA content. As shown in Fig. 1, we observed that ethanol (100 mM) exposure for 24 h reduced the protein (Fig. 1A and B; ANOVA, $F(1,11)=15.00$; $P=0.0026$) and mRNA (Fig. 1C and D). ANOVA, $F(1,6)=15.15$; $P=0.0081$) levels on NAPE-PLD and this effect persisted at 48 h post-ethanol exposure (Fig. 1E and F). ANOVA $F(1,18)=23.28$; $P=0.0001$.).

3.2. Acute exposure to ethanol activates the cAMP/CREB signaling pathway

Several studies have shown that ethanol activates the cAMP/CREB signaling pathway in the brain (Asher et al., 2002; Asyyed et al., 2006; Constantinescu et al., 2004). Therefore, we sought to investigate whether this signaling pathway was activated by ethanol exposure in BV2 microglial cells. Firstly, we performed a time-course analysis of the changes in cAMP levels in response to ethanol (100 mM). We observed a rise in intracellular levels of cAMP as soon as 5 min post-exposure to ethanol. As shown in Fig. 2A (ANOVA, $F(5,31)=4.6$; $P=0.003$), these cAMP levels reached a plateau after 15 min post-ethanol exposure and remained elevated due to the presence of IBMX (800 μM), a phosphodiesterase inhibitor.

cAMP-response-element-binding protein (CREB) is a cellular transcription factor that responds to cAMP levels and binds to DNA sequences called cAMP-response-elements (CRE). In order to study whether the increased levels of intracellular cAMP resulted in an increased activation of CREB, we performed a time-course western blot analysis of the levels of phosphorylation of this transcription factor. As shown in Fig. 2B and C (ANOVA, $F(5,42=19.13$; $P<0.0001$), the exposure to ethanol (100 mM) induced an increase in CREB phosphorylation, with a peak at 30 min and remained elevated up to 60 min post-stimulation.

cAMP-dependent protein kinase (PKA) is one of the kinases that activates CREB. Therefore, our next experiment was to test whether PKA was involved in the ethanol-induced phosphorylation of CREB. BV2 cells were pre-incubated with H-89 (10 μM), a PKA inhibitor, for 30 min before being exposed to ethanol (100 mM). As shown in Fig. 2D and E (ANOVA, $F(3,12)=24.84$; $p<0.0001$), inhibition of PKA reversed to control levels the ethanol-induced phosphorylation of CREB.

3.3. Acute exposure to ethanol activates the ERK1/2 MAPK signaling pathway

Several reports indicate that ethanol consumption increases ERK1/2 phosphorylation in the brain (Groblewski et al., 2011; Peana et al., 2013; Rosas et al., 2014). Therefore, we sought to study whether acute ethanol exposure could activate the microglial ERK1/2 signaling pathway. We exposed BV2 cells to ethanol (100 mM) at different times and analyzed by western blot the phosphorylation of ERK1/2 MAPK. As shown in Fig. 3A and B (ANOVA, $F(5,18)=5.73$; $P=0.0025$), the exposure to ethanol induced a time-dependent increase in pERK1/2, with a peak at 30 min that remained elevated up to 60 min post-stimulation. Next, we decided to investigate whether the ethanol-induced activation of ERK1/2 was involved in CREB phosphorylation. BV2 cells were pre-incubated with PD98059 (10 μM) for 30 min before being exposed to ethanol (100 mM). As shown in Fig. 3C and D (ANOVA, $F(3,12)=5$; $P=0.0178$), inhibition of activation of ERK1/2 reversed to control level the ethanol-induced phosphorylation of CREB.

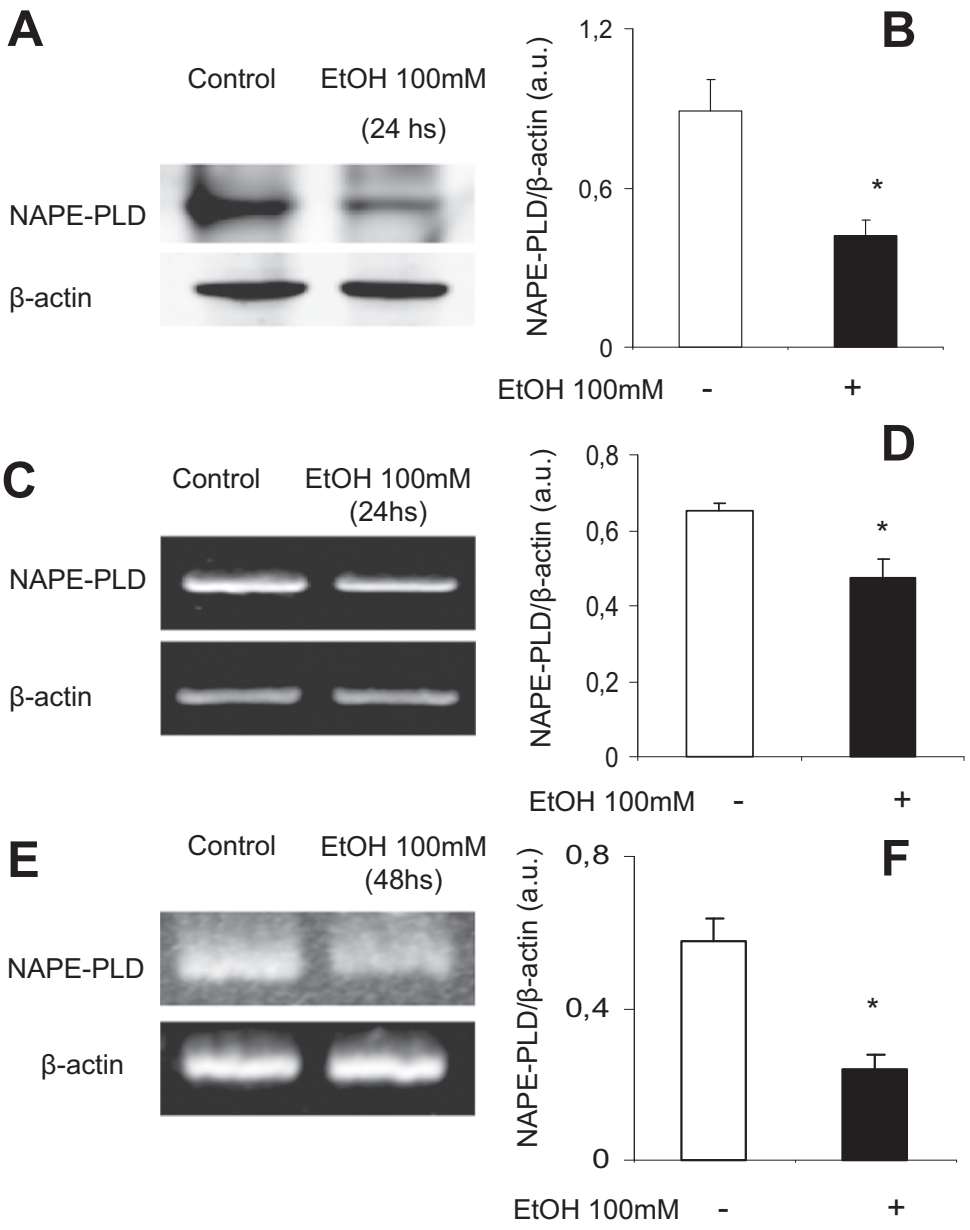

Fig. 1. Ethanol downregulates microglial expression of NAPE-PLD. (A and B) Murine BV2 microglial cells were exposed for 24 h to ethanol (EtOH, 100 mM) after which protein was collected and NAPE-PLD expression was assessed by western blot and normalized to β-actin expression (NAPE-PLD/β-actin). Statistics: * vs. control. $P=0.0026$. (C and D) Murine BV2 microglial cells were exposed for 24 h to ethanol (100 mM) after which total mRNA was collected and NAPE-PLD mRNA expression was assessed by RT-PCR and normalized to β-actin mRNA expression (NAPE-PLD/β-actin). Statistics: * vs. control. $P=0.0081$. (E and F) Murine BV2 microglial cells were exposed for 48 h to ethanol (100 mM) after which total mRNA was collected and NAPE-PLD mRNA expression was assessed by RT-PCR and normalized to β-actin mRNA expression (NAPE-PLD/β-actin). Statistics: * vs. control. $P=0.0001$.

3.4. Acute exposure to ethanol upregulates histone acetyl transferase activity via cAMP/PKA and ERK1/2 signaling pathways in BV2 cells

Changes in the pattern of acetylation and/or methylation of histones are involved in gene expression/repression. Ethanol consumption has been associated with epigenetic changes in the brain (Caputi et al., 2015; Pascual et al., 2012; Zou and Crews, 2014), particularly with changes in the acetylation pattern of histone 3 (H3) (Aroor et al., 2010; Kim and Shukla, 2006; Park et al., 2003; Qiang et al., 2011). Therefore, we sought to investigate whether acute ethanol exposure to BV2 cells modified the acetylation pattern of H3 and whether it had an effect on the histone acetyl transferases (HAT) activity. Firstly, we studied the effect of acute ethanol exposure to HAT activity in BV2 cells. As shown in Fig. 4A (ANOVA, $F(2,18)=11.55$; $P=0.0006$), we found that ethanol (100 mM) increased microglial HAT activity at 12 h post-exposure and that this effect remained elevated at 24 h post-stimulation. This increased HAT activity was translated in higher levels of acetylated H3 (Fig. 4B and C). ANOVA, $F(3,12)=16.16$; $P=0.0002$). Next, we assessed the involvement of cAMP/PKA signaling pathway in the ethanol-induced increase in HAT activity. We found that inhibition of PKA with H-89 (10 μM) decreased the ethanol-induced increase in HAT activity (Fig. 5A). ANOVA, $F(3,20)=8.29$; $P=0.0009$), which was accompanied by a restoration to control levels of the alcohol-increased acetylation of H3 (Fig. 5B and C). ANOVA, $F(3,12)=4.1$; $P=0.0323$). Finally, we studied the involvement of ERK1/2 MAPK signaling pathway in the ethanol-induced increase in HAT activity. We found that inhibition of ERK1/2 with PD98059 (10 μM) decreased the ethanol-induced increase in HAT activity (Fig. 6A). ANOVA, $F(3,20)=7.01$; $P=0.0021$). This reduction

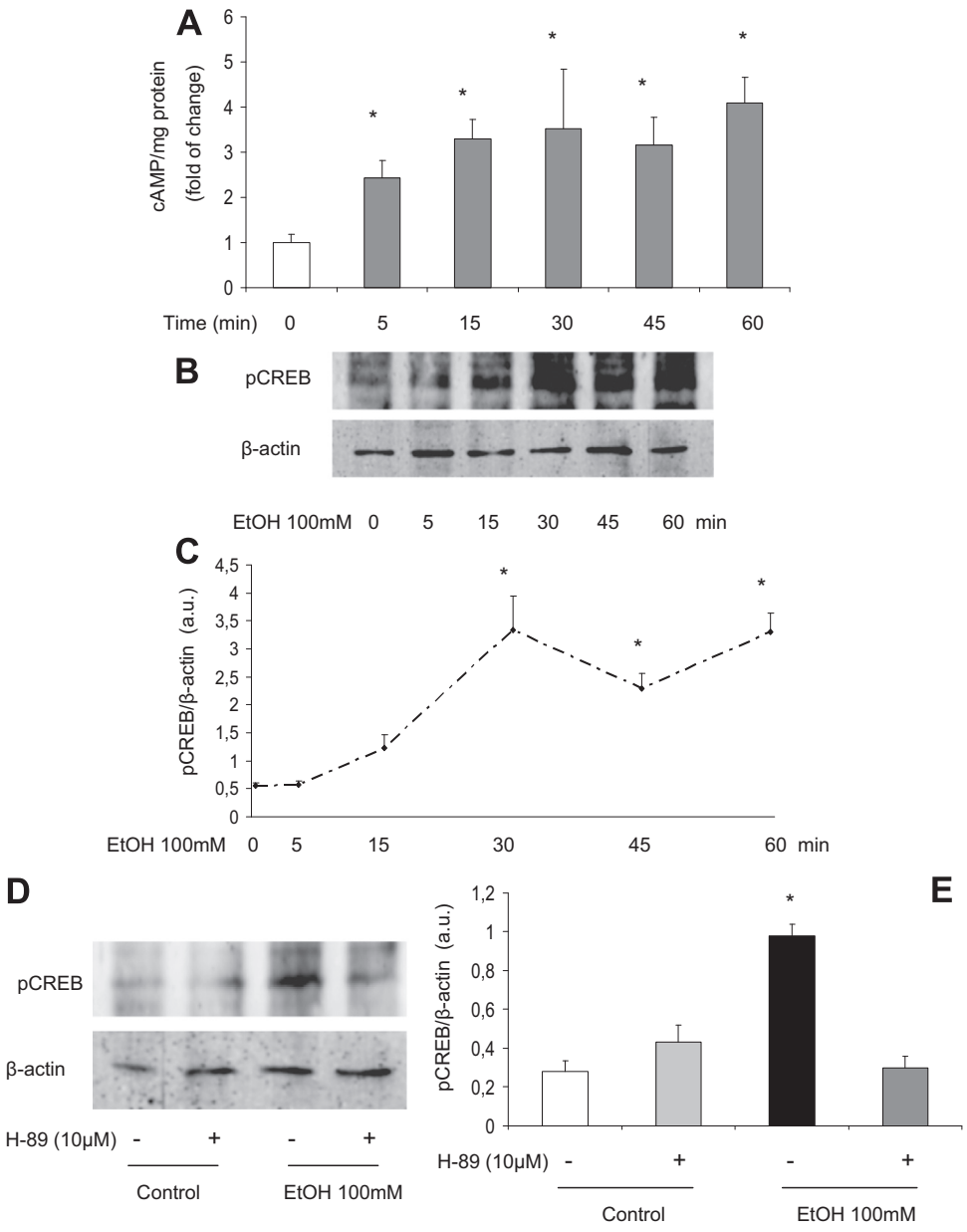

Fig. 2. Ethanol exposure activates microglial cAMP/CREB signaling pathway. (A) Time course changes in intracellular content of cAMP in murine BV2 microglial cells induced by exposure to ethanol (100 mM). Statistics: * vs. control. $P=0.003$. (B) Western blot and (C) densitometric analysis of the time course changes in CREB phosphorylation in murine BV2 microglial cells induced by exposure to ethanol (EtOH, 100 mM). Statistics: * vs. control. $P<0.0001$. (D) Western blot and (E) densitometric analysis of the participation of cAMP/PKA signaling pathway on ethanol-induced CREB activation. PKA inhibition with H-89 (10 μM) completely blocked the effect of ethanol (100 mM) on CREB phosphorylation. Statistics: * vs. control. $P<0.0001$. pCREB expression was normalized to β-actin expression (pCREB/β-actin).

in HAT activity was followed by a reversal to control levels of the alcohol-increased acetylation of H3 levels (Fig. 6B and C). ANOVA, $F(3,12)=4.27$; $P=0.0287$).

3.5. Both cAMP/PKA and ERK1/2 MAPK signaling pathways are involved in ethanol-induced downregulation of NAPE-PLD

Since both cAMP/PKA and ERK1/2 were signaling pathways activated by acute ethanol exposure, we decided to study their participation in the ethanol-induced downregulation of NAPE-PLD mRNA. Firstly, we performed a qPCR analysis using the PKA inhibitor, H-89 (10 μM). We observed that the effects of ethanol were reversed in the presence of H-89 and the NAPE-PLD mRNA levels were restored to control levels (Fig. 7A). ANOVA, $F(3,27)=5.53$; $P=0.0043$). Next, we performed a qPCR analysis using the inhibitor of ERK1/2 phosphorylation, PD98059 (10 μM). Similarly, we observed that the ethanol-induced downregulation of NAPE-PLD mRNA levels was reversed back to control levels (Fig. 7B). ANOVA, $F(3,20)=13.96$; $P<0.0001$).

4. Discussion

Ethanol and the endocannabinoid system (eCS) interact in a complex fashion. Several reports indicate that the eCS mediates ethanol acute effects and participates in the establishment of ethanol tolerance/dependence, propensity to relapse and its reinforcing effects (reviewed by Pava and Woodward (2012)). Conversely, acute ethanol administration has been shown to modify the endocannabinoid content as well as the expression of eCS components in different brain regions. Thus, Rubio et al. (2007) showed a reduction in anandamide content and changes in fatty

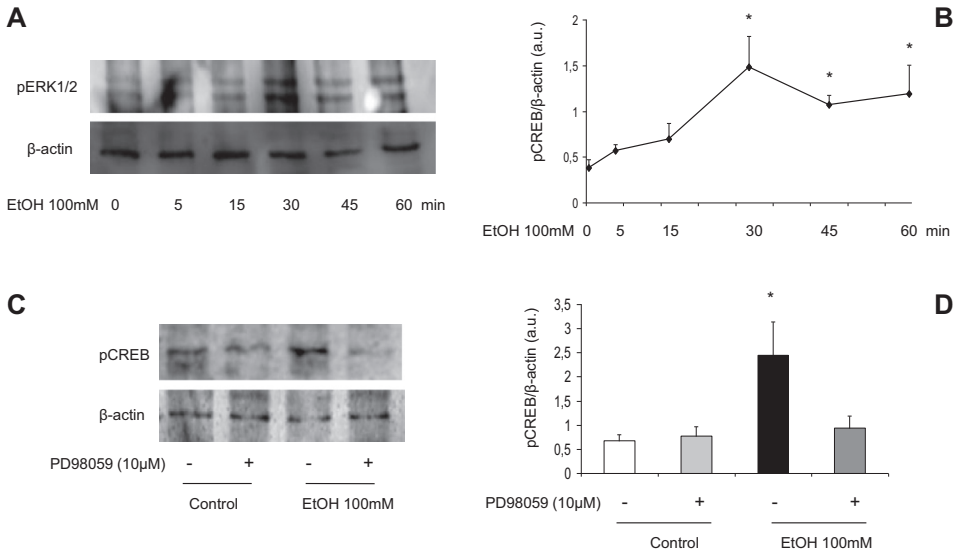

Fig. 3. Ethanol exposure activates microglial ERK1/2 MAPK signaling pathway. (A) Western blot and (B) densitometric analysis of the time course changes in ERK1/2 MAPK phosphorylation in murine BV2 microglial cells induced by exposure to ethanol (EtOH, 100 mM). Statistics: * vs. control. $P=0.0025$. (C) Western blot and (D) densitometric analysis of the participation of ERK1/2 MAPK signaling pathway on ethanol-induced CREB activation. MEK-1 inhibition with PD98059 (10 μM) completely blocked the effect of ethanol (100 mM) on CREB phosphorylation. Statistics: * vs. control. $P=0.0178$. pERK1/2 expression was normalized to β-actin expression (pERK1/2/β-actin).

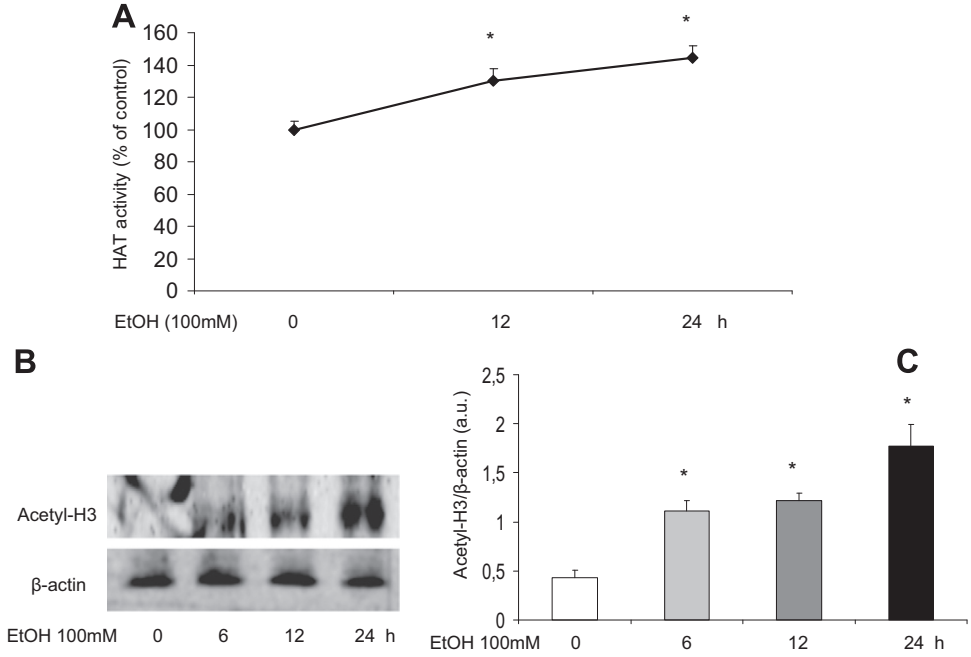

Fig. 4. Ethanol exposure increases microglial HAT activity and H3 acetylation. (A) Time course changes histone acetyltransferase (HAT) activity in murine BV2 microglial cells followed by exposure to ethanol (EtOH, 100 mM). Statistics: * vs. control. $P=0.0006$. (B) Western blot and (C) densitometric analysis of the time course changes in histone H3 acetylation (Acetyl-H3) in murine BV2 microglial cells induced by exposure to ethanol (100 mM). Statistics: * vs. control. $P=0.0002$. Acetyl-H3 expression was normalized to β-actin expression (Acetyl-H3/β-actin).

acid amide hydrolase (FAAH) activity in the hypothalamus, caudate-putamen and amygdala of acute ethanol exposed animals. Similarly, Ferrer et al. (2007) reported decreased anandamide levels in cerebellum, hippocampus and ventral striatum after in vivo acute administration of alcohol. These authors found no changes in FAAH activity and proposed that an altered NAPE-PLD activity was responsible for the lower anandamide content in those brain regions (Ferrer et al., 2007). Contrarily, Basavarajappa et al. (2008) showed that in vitro acute ethanol exposure to cultured hippocampal neurons increased anandamide formation. Therefore, ethanol could diversely affect the eCS of the different brain cell types. Here, we show that ethanol downregulates microglial NAPE-PLD expression via activating cAMP/PKA and ERK1/2 MAPK signaling pathways and increasing HAT activity.

Cumulative, although sometimes conflicting, evidence show that ethanol alters microglial phenotype and function. In this sense, Lee et al. (2004) failed to observe changes in the microglial expression of IL-1β and nitric oxide (NO) after ethanol exposure. Moreover, they showed that ethanol had an inhibitory effect on LPS activation of microglial cells. In contrast, Boyadjieva and Sarkar (2010) showed that ethanol treatment increased microglial release of TNF-α, IL-1β and IL-6 and that these cytokines as well as ethanol-induced increased ROS production were toxic to hypothalamic neurons in vitro (Boyadjieva and Sarkar, 2013).

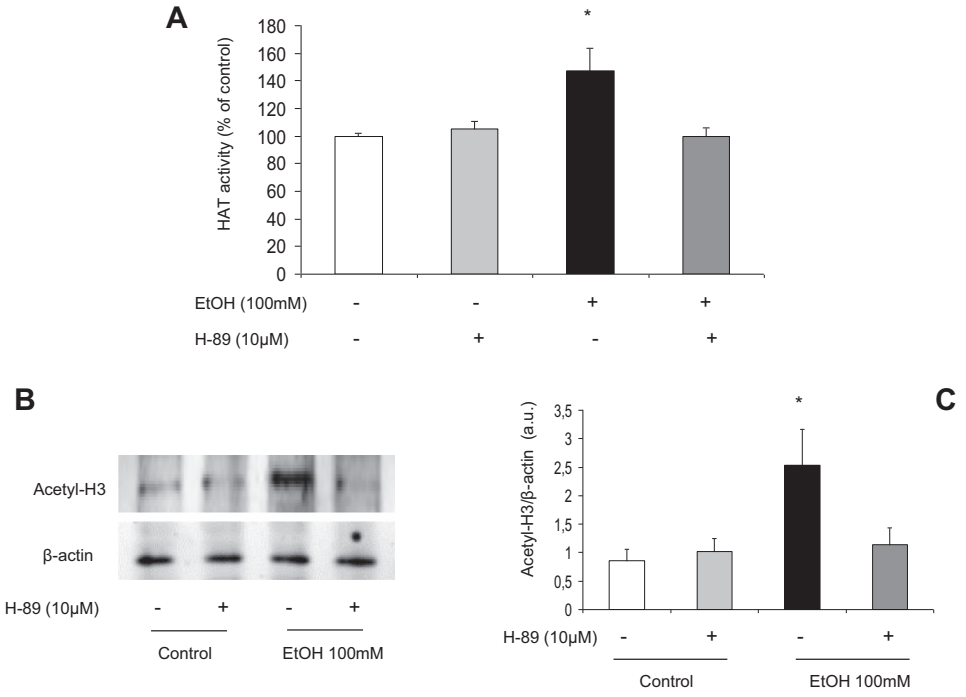

Fig. 5. Ethanol increases H3 acetylation via PKA signaling pathway. (A) Inhibition of cAMP/PKA signaling pathway with H-89 (10 μM) completely reversed the increased in HAT activity induced by ethanol (EtOH, 100 mM). Statistics: * vs. control. $P=0.0009$. (B) Western blot and (C) densitometric analysis of the participation of PKA signaling pathway on ethanol-induced histone H3 acetylation (Acetyl-H3). PKA inhibition with H-89 (10 μM) completely blocked the effect of ethanol (100 mM) on histone H3 increased acetylation. Statistics: * vs. control. $P=0.0323$. Acetyl-H3 expression was normalized to β-actin expression (Acetyl-H3/β-actin).

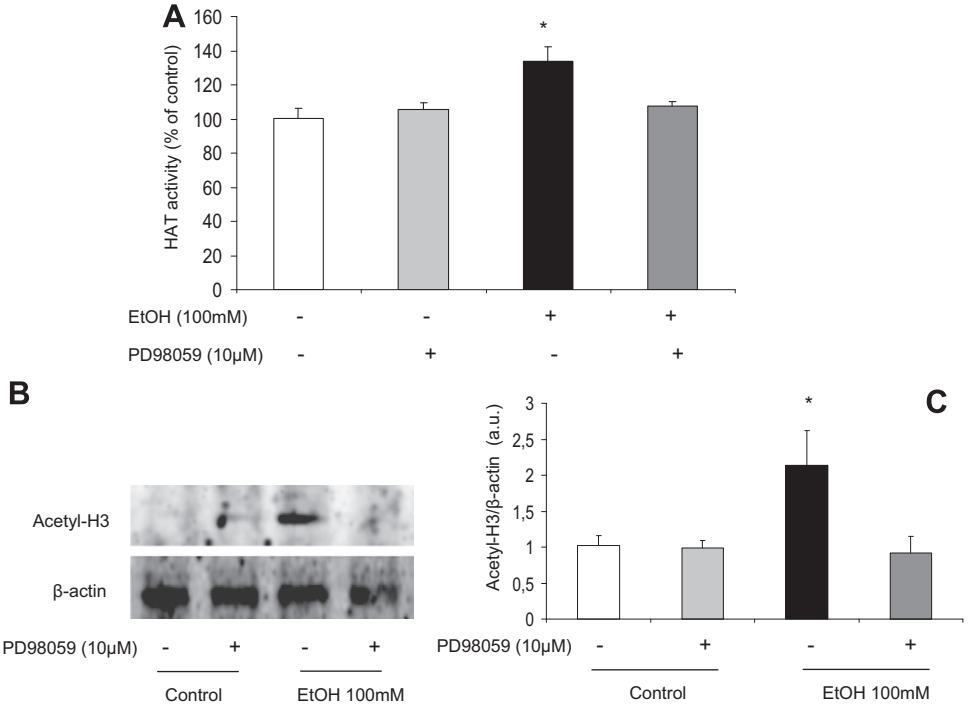

Fig. 6. Ethanol increases H3 via ERK1/2 MAPK signaling pathway. (A) Inhibition of ERK1/2 MAPK signaling pathway with PD98059 (10 μM) completely reversed the increased in HAT activity induced by ethanol (100 mM). Statistics: * vs. control. $P=0.0021$. (B) Western blot and (C) densitometric analysis of the participation of ERK1/2 MAPK signaling pathway on ethanol-induced histone H3 acetylation. MEK-1 inhibition with PD98059 (10 μM) completely blocked the effect of ethanol (100 mM) on histone H3 increased acetylation. Statistics: * vs. control. $P=0.0287$. Acetyl-H3 expression was normalized to β-actin expression (Acetyl-H3/β-actin).

Similarly, it has been reported that ethanol exposure increased microglial activation and production of TNF-α, IL-1β and NO in the culture medium (Fernandez-Lizarbe et al., 2013, 2009). cAMP/PKA signaling pathway has been shown to mediate ethanol effects in the brain as well as in cultured neurons (Asher et al., 2002; Asyyed et al., 2006; Constantinescu et al., 2004). These reports are in agreement with our observation that exposure of murine BV2 microglial cells to acute ethanol increased intracellular levels of

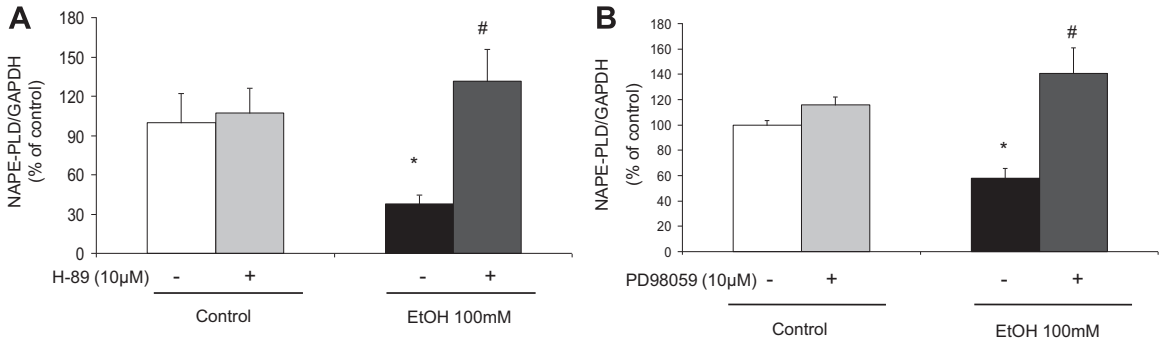

Fig. 7. PKA and ERK1/2 MAPK signaling pathways are involved in the effects of ethanol on NAPE-PLD mRNA expression. (A) Inhibition of cAMP/PKA signaling pathway with H-89 (10 μM) completely restored to control level the ethanol-induced downregulation of NAPE-PLD mRNA. Statistics: * vs. control; # vs. EtOH 100 mM. $P=0.0043$. (B) Inhibition of ERK1/2 MAPK signaling pathway with PD98059 (10 μM) completely reversed the effects of ethanol (EtOH, 100 mM) on NAPE-PLD mRNA expression. Statistics: * vs. control; # vs. EtOH 100 mM. $P<0.0001$. In both cases, NAPE-PLD mRNA expression was normalized to GAPDH mRNA expression (NAPE-PLD/GAPDH).

cAMP and, through PKA signaling pathway, CREB phosphorylation. Ethanol stimulates cAMP signaling via at least two different mechanisms (reviewed in Newton and Messing (2006)). The first one involves the inhibition of the membrane protein, equilibrative nucleoside transporter 1 (ENT-1), resulting in the extracellular accumulation of adenosine and activation adenosine receptor A2a (Nagy et al., 1990), which is coupled to a G_s protein and therefore stimulates cAMP synthesis and PKA activation (Mailliard and Diamond, 2004). The second one involves the stimulation of specific isoforms of adenylyl cyclases (AC), with AC7 being the most ethanol-sensitive isoform (Yoshimura and Tabakoff, 1995). In response to ethanol, neural cells increase cAMP production which activates PKA, which mediates CREB phosphorylation and eventually the induction of CRE-mediated gene expression (Asher et al., 2002; Constantinescu et al., 2004). Similarly, we observed that the increased phosphorylation of CREB in response to ethanol in BV2 microglial cells was mediated by PKA signaling pathway, since its inhibition with H-89 completely blocked ethanol's effects.

ERK1/2 MAPK signaling pathway has also been implicated as mediator of ethanol effects in cultured hepatocytes (Lee et al., 2002), the liver (Aroor et al., 2010) and the brain (Groblewski et al., 2011; Peana et al., 2013; Rosas et al., 2014). Similarly, we found that acute ethanol exposure to murine BV2 microglial cells increased the phosphorylation of ERK1/2 in a time-dependent fashion. Activated ERK1/2 translocates into the nucleus where it phosphorylates transcription factors as well as nucleosomal and chromatin proteins (reviewed by Delcuve et al., 2009). Interestingly, we observed that inhibition of ERK1/2 activation blocked the effect of ethanol on CREB phosphorylation, suggesting that ERK1/2 signaling pathway is required for CREB activation. This is in agreement with previous reports showing that ERK1/2 activates CREB by phosphorylating at serine residue 133 (Gee et al., 2006) and that phosphorylation of CREB via activated ERK1/2 is necessary for the neuroprotective effects of the tissue plasminogen activator on cultured neurons (Wu et al., 2013).

Once phosphorylated, CREB interacts with CREB binding protein (CBP) and p300 not only to modify the expression of their target genes but also to modulate the histone acetylation pattern (Kasper et al., 2010; Mayr and Montminy, 2001). Indeed, CBP and p300 have intrinsic histone acetyltransferase activity (Liu et al., 2008). Several lines of research have shown that ethanol epigenetically alters gene expression by increasing HAT activity and, consequentially inducing histone hyperacetylation. Thus, Park et al. (2005) showed that ethanol induced hyperacetylation at lysine residue 9 of histone 3 (H3) by increasing HAT activity in rat hepatocytes and that this effect was mediated by activated ERK1/2. Choudhury et al. (2011) reported that ethanol also induced histone acetylation by activating members of the GNAT family of HAT in human hepatoma cells. Kim and Shukla (2006) reported that acute ethanol administration to rats increased the acetylation at residue 9 of histone H3 in liver, lung, spleen and testes. Similarly, Pandey et al. (2008) have shown that acute ethanol consumption was associated with and increased acetylation of histone H3 and histone H4 as well as a higher expression of CBP in the amygdaloid brain regions of rats. These reports support our findings that acute ethanol exposure increased HAT activity in murine BV2 microglial cells and that this increased activity was mediated by activation of the cAMP/PKA and ERK1/2 signaling pathways. Moreover, both signaling pathways were necessary for the effects on ethanol in suppressing the mRNA expression of NAPE-PLD in murine BV2 microglial cells, since the inhibition of either pathway completely blocked the effects of ethanol.

Collectively, our results suggest that acute ethanol exposure to microglial cells changes the expression of the main synthesizing enzyme for anandamide, NAPE-PLD and that the molecular mechanisms involved include the cAMP/PKA and ERK1/2 signaling pathways as well as possibly epigenetic changes via increased HAT activity and histone H3 acetylation. It has been shown that NAPE-PLD knock-out mice are unable to mount a normal inflammatory reaction in response to carrageenan (Zhu et al., 2011). Therefore, further research is warranted to understand the physiological relevance for the downregulation of NAPLE-PLD expression for microglial cell function and/or for the pathophysiology of ethanol intoxication.

Conflict of interests

The authors have no conflict of interests to declare.

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