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Postnatal ethanol exposure alters levels of 2-arachidonylglycerol-metabolizing enzymes and pharmacological inhibition of monoacylglycerol does not cause neurodegeneration in neonatal mice

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Abstract

The consumption of ethanol by pregnant women may cause neurological abnormalities, affecting learning and memory processes in children, and are collectively described as fetal alcohol spectrum disorders (FASDs). However, the molecular mechanisms underlying these changes are still poorly understood. In our previous studies, we found that ethanol treatment of postnatal day 7 (P7) mice significantly enhances anandamide (AEA) levels but not 2-arachidonylglycerol (2-AG) levels and induces widespread neurodegeneration, but the reason for the lack of significant effects of ethanol on the 2-AG level is unknown. In this study, we examined developmental changes in diacylglycerol lipase-α, β (DAGL-α and β) and monoacylglycerol lipase (MAGL). We found that the levels of these proteins were significantly higher in adult brains compared to those detected early in brain development. Next, we examined the influence of P7 ethanol treatment on these enzymes, finding that it differentially altered the DAGL-α protein and mRNA levels but consistently enhanced those of the DAGL-β. Interestingly, the ethanol treatment enhanced MAGL protein and mRNA levels. Inhibition of MAGL with KML29 failed to induce neurodegeneration in P7 mice. Collectively, these findings suggest that ethanol significantly activates DAGL-β and MAGL in the neonatal brain, resulting in no net change in 2-AG levels.

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Keywords

neurodegeneration; DAGL-α/β; MAGL; anandamide; cannabinoids; development; CB1R; fetal alcohol spectrum disorder (FASD)

Introduction

Fetal alcohol spectrum disorder (FASD) is an umbrella term used to describe a range of neurological defects that can occur in an individual whose mother abused alcohol during pregnancy (Feldman *et al.* 2012, Lewis *et al.* 2012, Paintner *et al.* 2012a, Paintner *et al.* 2012b). A recent survey has suggested that the present prevalence of FASD in the United States and several Western European countries may be as high as 2-5% (May *et al.* 2009). The increase in the incidence of FASD is a major factor underlying the rises in the number of children and adults with learning disabilities and cases of non-genetic intellectual disability in Western nations (Morleo *et al.* 2011). FASD is associated with widespread neuropsychological manifestations (Mattson & Riley 1998, Mattson *et al.* 1998), such as reduced intellectual ability and behavioral problems (Goodman *et al.* 1999, Harris *et al.* 1995, Mattson *et al.* 2011, Mattson *et al.* 1999, Rasmussen *et al.* 2006). In mice, a significant proportion of third trimester-equivalent (Bayer *et al.* 1993) brain development takes place following birth (Cronise *et al.* 2001, Tran *et al.* 2000), and rapid synaptic growth occurs during postnatal days 4-10 (P4-10). Therefore, in binge ethanol models, ethanol has been directly administered to neonatal pups to examine the effects of ethanol consumption during the third trimester of fetal development in humans (Gil-Mohapel *et al.* 2010). The major immediate effect of single-day ethanol intoxication in P7 mice has been found to be the widespread activation of caspase-3 (Ikonomidou *et al.* 2000) in many brain regions including hippocampus (HP) and neocortex (NC), which are important for learning and memory (Kelly *et al.* 2009). This ethanol paradigm has been shown to cause persistent neurobehavioral abnormalities in adult mice (Noel *et al.* 2011, Sadrian *et al.* 2012, Subbanna & Basavarajappa 2014, Subbanna *et al.* 2015, Subbanna *et al.* 2013a, Wilson *et al.* 2011).

The effects of ethanol are mediated through several signal transduction pathways involving many neurotransmitters and ion channels in various brain regions, one of which is the endocannabinoid system (EC), which is comprised of endogenous cannabinoids, their receptors (cannabinoid receptors type 1 and 2; CB1R and CB2R), and the enzymes involved in their metabolism (Basavarajappa & Arancio 2008, Mechoulam & Parker 2013). The EC system regulates synaptic events via endocannabinoids, such as anandamide (AEA) and 2-arachidonylglycerol (2-AG) (Kreitzer & Regehr 2001, Ohno-Shosaku *et al.* 2001, Wilson & Nicoll 2001), in developing (Hansen *et al.* 2008, Harkany *et al.* 2008, Subbanna et al. 2015, Subbanna et al. 2013a) and adult brains (Mechoulam & Parker 2013). AEA, 2-AG and their G-protein-coupled receptor, CB1R, have been shown to be prime targets of ethanol sensitivity, tolerance and dependence in adult animals (Basavarajappa 2007, Basavarajappa *et al.* 2008, Basavarajappa *et al.* 2006, Hungund *et al.* 2003, Rubio *et al.* 2009), including humans (Ceccarini *et al.* 2014, Hirvonen *et al.* 2013, Marcos *et al.* 2012). There is strong evidence that ECs and CB1R have decisive functions in neuronal maturation during brain development (Bisogno *et al.* 2003, Fernandez-Ruiz *et al.* 2000).

In our previous studies, we have found enhanced CB1R expression and increased AEA but not 2-AG levels which lead to neurodegeneration in ethanol-treated P7 mice. Enhanced AEA levels in ethanol-treated P7 mice were achieved via transcriptional activation of enzymes involved in AEA biosynthesis. Blockade with CB1R antagonist or genetic deletion of CB1R provided protection against ethanol-induced neurodegeneration, suggesting that the brain response involves the specific activation of the AEA-CB1R pathway and the maintenance of the 2-AG mediated effects under normal conditions (Subbanna et al 2013a). The reason for the lack of ethanol effects on 2-AG levels is not known. Unlike the AEA level, the 2-AG level is two hundred-fold higher in the brain (Sugiura *et al.* 2006), and its biosynthesis is catalyzed by two diacylglycerol lipase isoforms (diacylglycerol lipase-α/β and DAGLα/β) (Bisogno et al. 2003). DAGL-α is expressed throughout the brain, but the expression pattern of DAGL-β has not been well characterized (Basavarajappa 2014, Oudin *et al.* 2011). *In vivo*, 2-AG biosynthesis appears to be carried out by these two DAGLs, but the isoforms that are actually functioning in normal brains and in those that are diseased still remains to be established (Murataeva *et al.* 2014). It is well accepted that monoacylglycerol lipase (MAGL) is the predominant enzyme responsible for degrading 2-AG during synaptic activities, but at least four other enzymes, including alpha-beta-hydrolase domain 6 and 12 (ABHD6 and 12), fatty acid amide hydrolase (FAAH) and cyclooxygenase-2 (COX-2), have shown to be involved in 2-AG degradation, depending on the cell type and tissue-specific conditions (Basavarajappa 2014, Murataeva et al. 2014). Based on this interesting fact, in the current study, we further assessed the means by which 2-AG is maintained in the steady state in ethanol-treated P7 mice by examining the transcription and translation of enzymes involved in 2-AG metabolism. Postnatal ethanol treatment differentially regulated the DAGL-α/β and MAGL enzymes, resulting in steady-state 2-AG levels in the hippocampus and neocortex of the P7 mice. Inhibition of MAGL with KML29 failed to induce neurodegeneration in P7 mice.

Methods

Animals and ethanol treatment

C57BL/6J (Jackson Laboratory, Bar Harbor, ME, USA) mice were generated from breeding colony at NKI and housed in groups under standard laboratory conditions (12 h light/12 h dark cycle), with food and water available ad libitum. Animal care and handling procedures followed the Institutional (NKI IACUC) and National Institutes of Health guidelines. An ethanol treatment paradigm, which has been previously shown to induce robust apoptotic neurodegeneration in P7 mice (Olney et al. 2002), was used in the current study. Half of the male and female pups in each litter were treated subcutaneously (s.c.) with saline, and the other half were treated with ethanol at P7 (based on the day of birth) (2.5 g/kg s.c. at 0 h and again at 2 h) as described previously by our laboratory (Subbanna & Basavarajappa 2014, Subbanna et al. 2014, Subbanna et al. 2013b). The mice were kept with the dams until the pups were sacrificed, and their brains were removed at 4–24 h after the first saline/ethanol injection.

Analysis of AEA and 2-AG levels by LC/MS

Hippocampus and neocortex tissues were dissected at 8 h after first dose of ethanol or saline injection, flash frozen and stored at -80°C. Hippocampus and neocortex tissue homogenates were subjected to LC–MS using the isotopic dilution procedure to measure AEA and 2-AG levels as described previously (Subbanna et al. 2013a). The standard curve was fitted with a quadratic equation, with the curve encompassing ranges of 0.5–50 ng for AEA and 50-2500 ng for 2-AG, and was processed similar to quality controls prepared with brain tissue extracts.

KML29 treatment

For the KML29 experiments, KML29 (Cayman Chemicals, Ann Arbor, Michigan) was dissolved in 10μl of ethanol followed by a few drops of Tween 80 (10 μl) and the volume was made up with sterile saline solution. Half of the male and female C57BL/6J mice pups in each litter were treated subcutaneously (s.c.) with vehicle and the other half were treated with KML29 solution (0-20 mg/kg) by s. c. injection at a volume of 5 μl/g body weight as described previously by our laboratory (Subbanna & Basavarajappa 2014, Subbanna et al. 2014, Subbanna et al. 2013b). The KML29 vehicle solution was injected as a control. The mice were kept with the dams until the pups were sacrificed, and their brains were removed at 4–24 h after the KML29 injection.

Protein extraction, electrophoresis and immunoblotting

Four to 24 h after the first saline or ethanol injection, pups were sacrificed by decapitation, and the cortex and hippocampus were dissected, flash frozen and stored at -80°C. Hippocampus and neocortex tissue homogenates containing a freshly added 1% protease inhibitor mixture (Roche, Indianapolis, IN, USA) and phosphatase inhibitors were centrifuged at 7700 *g* for 1 min, and the supernatant (total extract) was aspirated. The membrane and cytosolic fractions were prepared from the total extract as described previously (Basavarajappa & Hungund 2001) and stored at -80°C until use. The samples for electrophoresis were prepared in a sample buffer as previously described by our laboratory (Basavarajappa et al. 2008). Blots were stained with Ponceau S to confirm equal loading in each lane and were incubated with a primary antibody, such as anti-rabbit MAGL (polyclonal #ab24701, 1:1000, Abcam, Cambridge, MA, USA), anti-goat DAGL-α (polyclonal #46-829, 1:1000), anti-rabbit-DAGL-β (1:1000; Kind gift from Dr. Ken Mackie, Indian University, Bloomington, IN), or anti-mouse-β-actin (monoclonal, catalog #3700, Cell Signaling), for 3 h at room temperature (22°C) or overnight at 4°C and processed as previously described by our laboratory (Basavarajappa et al. 2008). The incubation of the blots with a secondary antibody (goat anti-mouse peroxidase conjugate, #AP 124P, 1:5000 or goat anti-rabbit, #AP132P, 1:5000, Millipore, Billerica, MA, USA) alone resulted in no protein bands.

Real-time quantitative polymerase chain reaction (qPCR)

Four to 24 h after the first saline or ethanol injection, total RNA from the hippocampus and cortex samples was isolated using an RNeasy Mini Kit (Qiagen, Valencia, CA). mRNA was reverse-transcribed into complementary deoxyribonucleic acid (cDNA) using a Maxima

First Strand cDNA Synthesis Kit from Fermentas in a programmable thermal cycler (PCR-Sprint, Thermo Electron, Milford, MA). qPCR for the amplification of Faah, Dagl-α, β, Magl, Abdh4, Abdh6 and Abdh12 was performed with an integrated thermocycler and fluorescence detector (ABI PRISM 7900HT Sequence Detector; Applied Biosystems) using TaqMan® Gene Expression Assays Mm00813830_m1 (Dagl-α), Mm00523381_m1 (Dagl-β), Mm00449274_m1 (Magl), Mm00506368_m1 (Abdh4), Mm00481199_m1 (Abdh6), Mm00470489_m1 (Abdh12) and 4352932 (Gapdh) (Applied Biosystems). Glyceraldehyde3-phosphate dehydrogenase (GAPDH) was used as an endogenous mRNA control. For each run, triplicate reactions were carried out for each sample. Three independent experiments were carried out for each set of samples. Data were analyzed using SDS2.4 software (Applied Biosystems). The amount of target (Faah, Dagl-α, Dagl-β, Magl, Abdh4, Abdh6 and Abdh12), normalized to the endogenous reference (Gapdh) and relative to a calibrator was calculated with the equation -2ΔΔCt (Subbanna et al. 2014, Subbanna et al. 2013a, Subbanna et al. 2013b).

Statistical analysis

All of the data are presented as the mean ± SEM. A statistical comparison of the data was performed by one-way analysis of variance (ANOVA) with Bonferroni's post hoc test. For all comparisons, a $p < 0.05$ was considered statistically significant. Statistical analyses were performed using Prism software (GraphPad, San Diego, CA).

Results

P7 ethanol treatment induces caspase-3 activation and specifically enhances AEA levels without altering 2-AG levels in hippocampus and neocortex tissues

The exposure of P7 mice to the ethanol (2.5 g/kg, s.c. at 0 h and again at 2 h) treatment resulted in a BEL of 0.43 ± 0.34 g/dl at 3 h that was gradually reduced to 0.23 ± 0.06 g/dl at 9 h after the first ethanol injection. This treatment resulted in the widespread activation of capsase-3, as measured using a cleaved caspase-3 (CC3) antibody throughout the forebrain. Measurements were made at 8 h after the first dose of ethanol or saline [hippocampus ($F_{1, 11} = 55$, $p < 0.05$) and cortex ($F_{1, 11} = 120$, $p < 0.05$) regions] in the ethanol-exposed P7 brains (one-way ANOVA with Bonferroni's post hoc test) (Fig. 1a). We also measured CC3 protein levels by Western blot in hippocampal and neocortical protein cytosolic extracts. The results suggested that at 8 and 24 h after the first dose of ethanol, CC3 protein levels were significantly enhanced in both the hippocampus ($F_{3, 28} = 66$, $p < 0.05$) and neocortex ($F_{3, 28} = 55$, $p < 0.05$) (Fig. 1b) regions compared to those detected at 0 h (saline control) (one-way ANOVA with Bonferroni's post hoc test). The ethanol treatment significantly enhanced AEA but not 2-AG levels ($p > 0.05$) in both the hippocampus ($F_{3, 28} = 26$, $p < 0.05$) and neocortex ($F_{3, 28} = 25$, $p < 0.05$) tissues, as measured at 8 h after the first dose of ethanol or saline (Table 1). These observations suggest that ethanol-treatment of P7 mice recapitulated the previous findings on endocannabinoid levels and neurodegenerative conditions.

Ethanol exposure of P7 mice affects transcription and translation of 2-AG biosynthetic enzymes

Prior to the examination of the events responsible for the lack of effects of ethanol on 2-AG formation in P7 mice, we determined the developmental patterns of the DAGL-α and β proteins using cortical extracts. The levels of these proteins dramatically increased from the early postnatal to adult stages ($F_{7, 40} = 102$, $p < 0.05$) (Fig. 2). The levels of the housekeeping protein actin did not change significantly during the various stages of brain development, as shown previously (Jacob *et al.* 2011, Subbanna et al. 2013a, Subbanna et al. 2013b). We then determined whether ethanol affects the two major enzymes responsible for 2-AG biosynthesis (Basavarajappa 2014, Murataeva et al. 2014) and examined DAGL-α and β protein levels by Western blot analysis. Our results suggested that ethanol significantly reduced DAGL-α protein levels in the hippocampus ($F_{3, 28} = 15$, $p < 0.05$) but not in the neocortex ($F_{3, 28} = 1$, $p > 0.05$) (Fig. 3a) (one-way ANOVA with Bonferroni's post hoc test). We also measured DAGL-α mRNA levels. The results indicated mRNA levels were similar to the observed protein levels in the hippocampus ($F_{3, 28} = 15$, $p < 0.05$)) and neocortex (Fig. 3b) ($F_{3, 28} = 1.2$, $p > 0.05$) (one-way ANOVA with Bonferroni's post hoc test). Next, we measured DAGL-β protein levels. Our results suggested that ethanol significantly enhanced the levels of this protein in the hippocampus ($F_{3, 20} = 19$, $p < 0.001$) and neocortex ($F_{3, 20} = 27$, $p < 0.001$) (Fig. 4a) (one-way ANOVA with Bonferroni's post hoc test). Further, we determined DAGL-β mRNA levels to examine the transcriptional activation of the DAGL-β gene by the P7 ethanol treatment. Consistent with the protein levels, the mRNA levels were also significantly increased by ethanol in the hippocampus ($F_{3, 28} = 13$, $p < 0.05$) and neocortex ($F_{3, 28} = 12$, $p < 0.05$) at 8 and 24 h (Fig. 4b) (one-way ANOVA with Bonferroni's post hoc test). Together, these findings imply that although ethanol significantly enhanced 2-AG biosynthetic enzymes, especially DAGL-β, no significant increase in the 2-AG level occurred, which suggests that this endocannabinoid may be undergoing degradation.

Ethanol treatment of P7 mice specifically enhances transcription and translation of MAGL enzyme

As discussed earlier, 2-AG degradation is carried out by more than one degradative enzyme (Basavarajappa 2014, Murataeva et al. 2014). Thus, we screened the mRNA levels of the following enzymes: ABHD4, ABHD6, ABHD12, FAAH and MAGL. The results suggested that there were no significant increases in the ABHD4, ABHD6, ABHD12 or FAAH mRNA levels in the hippocampus (Fig. 5a) or neocortex (Fig. 5b) tissues ($p > 0.05$), as measured at 8 h after the first dose of ethanol or saline. However, MAGL mRNA levels were significantly increased in the hippocampus ($F_{3, 28} = 23$, $p < 0.05$) and neocortex ($F_{3, 28} = 10$, $p < 0.05$) tissues (one-way ANOVA with Bonferroni's post hoc test) (Fig. 5a and b). Prior to analysis of the effects of ethanol on MAGL protein levels, we determined the developmental pattern of the MAGL protein using cortical extracts. The levels of this protein dramatically increased from the early postnatal to adult stages ($F_{7, 40} = 287$, $p<0.05$) (Fig. 6a). The levels of the housekeeping protein actin did not change significantly during the various stages of brain development, as shown previously (Jacob et al. 2011, Subbanna et al. 2013a, Subbanna et al. 2013b). We then examined whether ethanol treatment affects MAGL protein levels in P7 mice. Our Western blot analyses suggested that ethanol significantly enhanced

the levels of this protein in both the hippocampus ($F_{3, 28} = 14$, $p < 0.05$) and neocortex ($F_{3, 28} = 45$, $p < 0.05$) (Fig. 6b) tissues. We also examined MAGL mRNA levels to determine whether ethanol affected the transcriptional activity of the MAGL gene in a time-dependent manner in P7 mice. Consistent with the protein levels, ethanol also enhanced the mRNA levels of this protein in the hippocampal ($F_{3, 28} = 58$, $p < 0.05$) and cortical ($F_{3, 28} = 12$, $p < 0.05$) (Fig. 6c) regions. Thus, although ethanol persistently enhanced the transcription and translation of DAGL-β, the simultaneous transcriptional activation of MAGL led to the normalization of the 2-AG levels in the P7 mice.

Inhibition of MAGL by KML29 failed to activate caspase-3 in P7 mice

To explore whether the inhibition of MAGL activity in vivo induces activation of caspase-3, we injected P7 mice with low to high dose of KML29 for up to 24 h. The results suggested that there were no significant increases in the cleaved caspase-3 levels in neocortex (Fig. 7) tissue ($p > 0.05$), as measured at 4- 24 h after the KML29 or vehicle treatment. In previous studies, KML29 has been shown to enhance 2-AG levels in mice at the doses used (Ignatowska-Jankowska *et al.* 2014, Kinsey *et al.* 2013, Schlosburg *et al.* 2010). Thus, inhibition of MAGL by KML29 which was shown to elevate endogenous levels of 2-AG failed to induce neurodegeneration in P7 mice.

Discussion

Here, we showed the means by which the postnatal ethanol treatment achieved enhanced AEA over 2-AG levels in the neonatal mouse brain. This precise on-demand local increase in AEA over 2-AG is attained through transcriptional and translational activation of well-characterized biosynthetic and degradative enzymes. In P7 ethanol-treated mice, AEA biosynthesis was enhanced through the transcriptional and translational upregulation of the N-arachidonoyl phosphatidylethanolamine-phospholipase D (NAPE-PLD) and glycerophosphodiesterase (GDE1) enzymes, but the catabolizing enzymes (ABHD4 and FAAH) were not altered (Subbanna et al. 2013a). In addition, although the P7 ethanol treatment consistently enhanced 2-AG biosynthetic enzymes, such as DAGL-β, also enhanced 2-AG catabolizing enzymes, such as MAGL, resulting in no significant change in the 2-AG levels. While these events are not economical to cells overall function, these observations suggest that the enhancement of AEA over 2-AG-CB1R pathway constitutes an example of the generation of unique cellular responses depending on the environment of the cell. Although our current and previous observations are in agreement with the suggested roles of endocannabinoids as modulators of neural cell fate (Berrendero *et al.* 1999, Fernandez-Ruiz et al. 2000, Rodriguez de Fonseca *et al.* 1993, Romero *et al.* 1997, Subbanna et al. 2015, Subbanna et al. 2013a) and with their ability to modulate signal transduction pathways that are essential for the regulation of cell fate in general (Fernandez-Ruiz et al. 2000, Mato *et al.* 2003, Rodriguez de Fonseca et al. 1993, Subbanna et al. 2015, Subbanna et al. 2013a), our present data provide the transcriptional and translational means through which postnatal ethanol was able to achieve specific AEA-CB1R-mediated ERK1/2/pCREB/Arc signaling (Subbanna et al. 2015, Subbanna et al. 2013a), rather than 2-AG mediated events in ethanol-induced neurodegeneration in the neonatal mice.

Although both AEA and 2-AG have been detected from the fetal period, the concentration of AEA has been found to be 1000-fold lower than that of 2-AG (Fernandez-Ruiz et al. 2000). Likewise, the developmental patterns significantly differ between the two endocannabinoids. AEA concentrations gradually increase throughout development to the adult stage (Berrendero et al. 1999), whereas 2-AG levels remain same throughout ontogeny, with a remarkably distinct peak on the first day after birth in rats. However, DAGL-α, β and MAGL expression levels are lower during early development and gradually increase throughout development to adulthood. Although the 2-AG concentration in the rodent brain is in the nanomolar range, which is one hundred times higher than that of AEA, it is 20 times less potent than AEA (Mechoulam *et al.* 1995). DAGL-α and β both contribute substantially to the regulation of steady-state levels of 2-AG in the brain and other tissues. However, in mice lacking DAGL-α, 2-AG levels are reduced by up to 80% in the brain and up to 50% in the DAGL-β null mouse brain (Gao *et al.* 2010). The manner by which ethanol preferably activates DAGL-β over DAGL-α even though both DAGL- α and β are less abundant in the developing brain compared to the adult brain (Bisogno et al. 2003) are unknown and warrant further investigations. Nonetheless, this work is still in its infancy, and future studies should address the mechanism by which AEA and 2-AG signaling is regulated in the developing brain.

While ethanol in the developing brain led to significantly increased levels of DAGL-β compared with those of DAGL-α, the net increase in 2-AG levels was not altered due to the ethanol-induced increase in the 2-AG-degrading enzyme MAGL. MAGL is a serine hydrolase that preferentially hydrolyzes 2-AG into glycerol and fatty acids, and it is the most highly expressed in the brain (Dinh *et al.* 2002). In our present study, we observed that the MAGL protein level was significantly lower during early brain development compared to that in the adult mouse brain. MAGL null mice or those with the pharmacological inhibition of MAGL exhibit elevated levels of 2-AG in the brain, and MAGL has been shown to be the primary enzyme responsible for 2-AG degradation in the brain (Chanda *et al.* 2010, Long *et al.* 2009, Pan *et al.* 2011, Saario *et al.* 2005, Schlosburg et al. 2010). However, ABHD6 and ABHD12, which show lower brain 2-AG serine hydrolase activity (approximately 5 and 9%, respectively) (Blankman *et al.* 2007), are not altered in the hippocampus or neocortex tissues of P7 mice exposed to ethanol. The ethanol treatment of P7 mice significantly enhanced MAGL protein levels in both the hippocampus and neocortex, which might have caused the immediate degradation of the excess 2-AG. These findings may also represent one of the reasons for the unaltered levels of 2-AG in the ethanol-treated P7 mouse brain. Therefore, ethanol reorganizes 2-AG metabolism through the transcriptional activation of synthesizing and degrading enzymes in the developing brain, facilitating the activation of AEA-CB1R over 2-AG/CB1R events, leading to neurodegeneration in the P7 mouse brain (Subbanna et al. 2015, Subbanna et al. 2013a). As DAGL and MAGL enzymes are distinctly localized at the neuroanatomical levels (Katona *et al.* 2006, Yoshida *et al.* 2006), local alterations of 2-AG and their influence on CB1R pathway cannot be ruled out from the current findings.

Several lines of evidence suggest that the origin of neurodevelopmental psychiatric disorders caused by stress or other environmental insults involve alterations in endocannabinoid metabolizing enzymes in the brain. In contrast with our observations of the ethanol-exposed

P7 mice, the exposure of neonatal P9 rats to a single prolonged 24-h episode of maternal deprivation significantly enhances DAGL-α without affecting DAGL-β immunoreactivity in the P13 hippocampus. In the same model, MAGL mRNA levels are reduced (Suarez *et al.* 2010). Another study has revealed that the exposure of P10 mice to a low dose of chlorpyrifos for 7 days significantly enhances the accumulation of AEA in the forebrain through the inhibition of FAAH. This treatment has no effect on 2-AG or MAGL (Carr *et al.* 2014). An additional study has found the accumulation of AEA and its associated lipid families, such as N-acylethanolamines (NAEs), in P7 rats exposed to an NMDA or NMDA antagonist or head trauma (Hansen *et al.* 2001). However, 2-AG and its associated lipid families, such as 2-monoacylglycerols (2-MAGs), are virtually unaffected. These three models have been well characterized to induce widespread caspase-3 activation in neonatal rodents (Hansen et al. 2001, Ikonomidou et al. 2000, Ikonomidou *et al.* 1999, Subbanna et al. 2013a). Although no biosynthetic enzymes have been evaluated in these studies, their findings suggest that even neurotoxic insults occurring during brain development significantly activate AEA/CB1R over 2-AG/CB1R events by regulating specific metabolic pathways that are required for normal brain development (Alpar *et al.* 2014, Berghuis *et al.* 2007, Keimpema *et al.* 2013a, Keimpema *et al.* 2013b, Keimpema *et al.* 2011, Keimpema *et al.* 2013c, Tortoriello *et al.* 2014). Further, DAGL-α and MAGL expression are reduced in the hippocampus of adolescent mice treated with valproic acid on gestational day 12.5. 2-AG levels are not altered by this regimen, but the animals exhibit autistic behavior (Kerr *et al.* 2013). Another study has shown that the expression levels of both DAGL-α and MAGL are be enhanced in FMR null mice (fragile X mental retardation syndrome 1 homolog) (Jung *et al.* 2012, Maccarrone *et al.* 2010), a model of fragile X syndrome, which is the most common genetic form of autism. In our previous studies, the pre-administration of a CB1R antagonist before P7 ethanol treatment or the use of CB1R null mice has been shown to prevent P7 ethanol-induced long-lasting neurobehavioral abnormalities, including spatial and social interaction memories, in adult mice (Subbanna et al. 2015, Subbanna et al. 2013a). Together, these observations suggest that alterations in the metabolism of specific endocannabinoids and their signaling pathways during the critical period of brain development cause long-lasting behavioral abnormalities in adulthood. In our study, treatment of P7 mice with MAGL inhibitor (KML29) failed to induce activation of caspase-3. JZL184 and its analog KML29 show good selectivity (>100-fold) for MAGL over FAAH (Fowler 2012, King *et al.* 2009, Mulvihill & Nomura 2013) and most other serine hydrolases (Chang *et al.* 2012, Long et al. 2009). The doses used were shown to inhibit MAGL and enhance brain levels of 2-AG in animals (Ignatowska-Jankowska et al. 2014, Kinsey et al. 2013, Schlosburg et al. 2010). Therefore, these observations suggest that 2-AG may not be involved in P7 ethanol-induced neurodegeneration but our study does not rule out the possible additional impact of 2-AG (both DAGL and MAGL enzymes) on the CB1R-mediated long-lasting effect on the brain function.

In summary, the ethanol treatment of P7 mice causes the specific up-regulation of AEA-CB1R signaling over the 2-AG-CB1R pathway by the specific reorganization of the enzymes that synthesize (DAGL-α/β) and degrade (MAGL) 2-AG. Further investigations of the roles of 2-AG during early brain development will be useful for understanding and

providing a novel pharmacological target for the treatment of behavioral traits associated with the neurodevelopmental origin of several brain diseases, including FASD.

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Abbreviations

AEA	anandamide
2-AG	2-arachidonylglycerol
CB	cannabinoid receptor
FASD	fetal alcohol spectrum disorder
DAGL	diacylglycerol lipase
MAGL	monoacylglycerol lipase

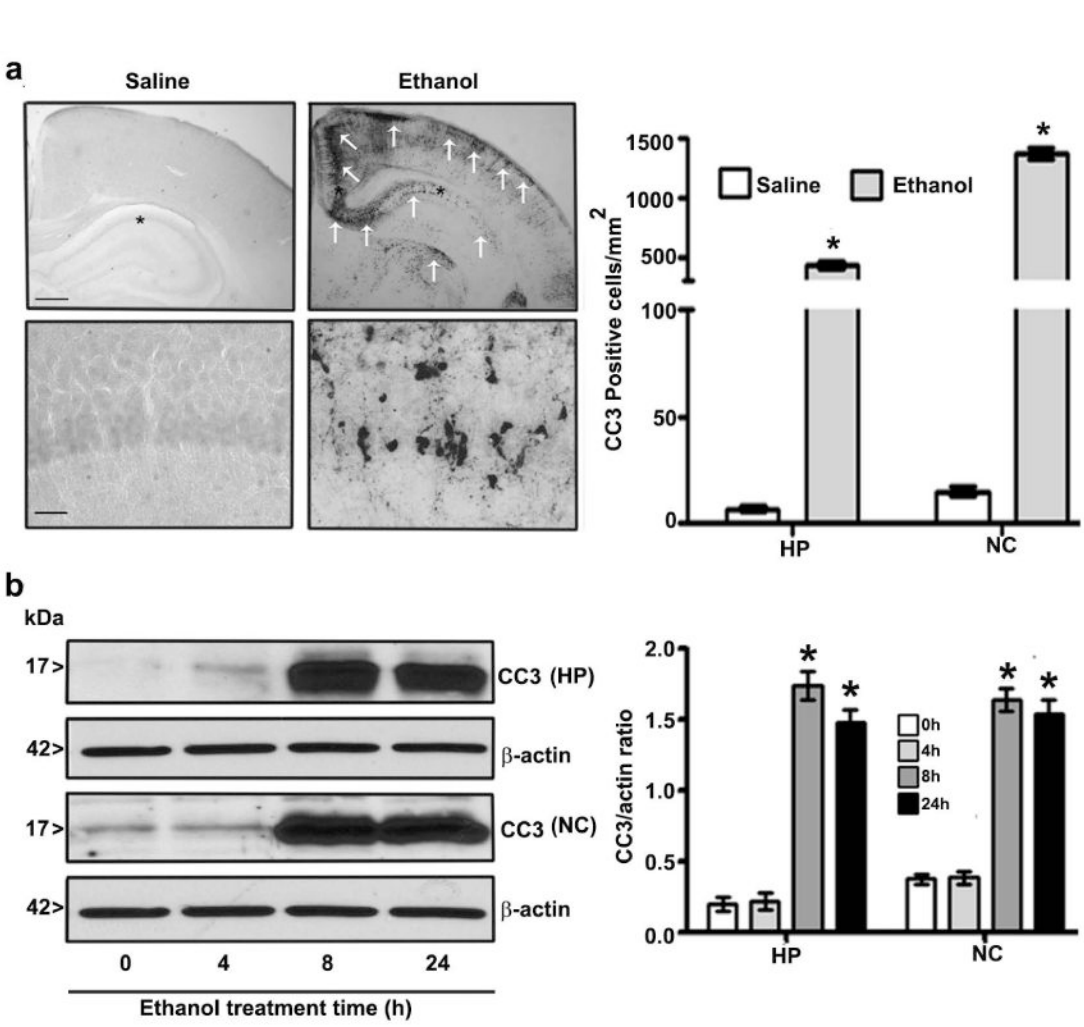

Fig. 1.

Ethanol exposure activates caspase-3 and alters endocannabinoid levels in the P7 mouse brain. (**a**) Coronal brain sections with hippocampus and retrosplenial cortex regions from saline- and ethanol-treated P7 mice were immunostained with an anti-rabbit CC3 antibody. CC3-positive neurons in the hippocampus and retrosplenial cortex are indicated by white arrows. Scale bars = 200 μm. Hippocampus and retrosplenial cortex images are enlarged to indicate CC3-positive cells (*). The scale bars represent 50 μm. CC3-positive neurons were counted in the hippocampus and the retrosplenial cortex (n = 8 pups/group). (**b**) CC3 levels were measured in cytosolic extracts of hippocampal and neocortical samples from the saline and ethanol groups (n = 15 pups/group) by Western blot analysis. β-actin was used as a loading control. HP, hippocampus; NC, neocortex.

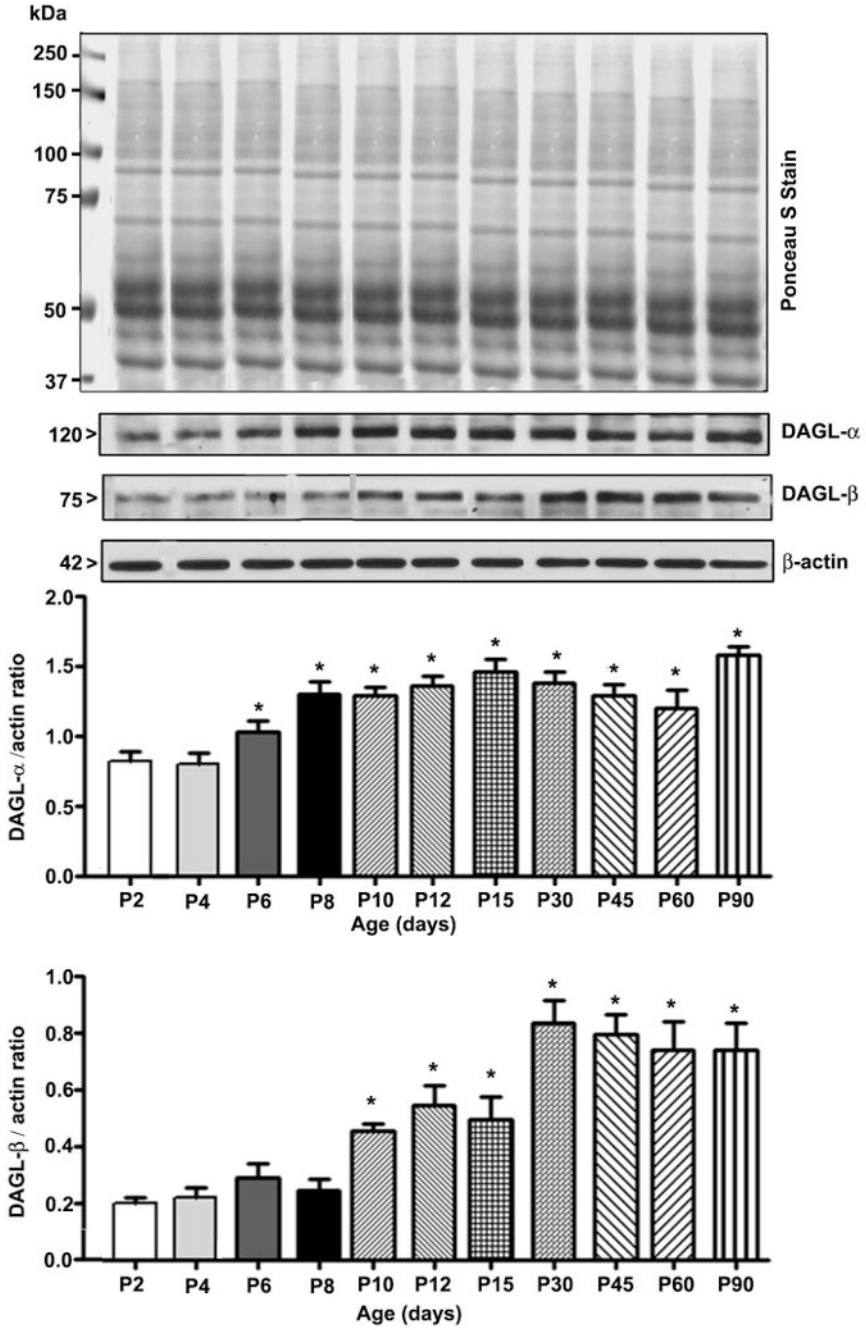

Fig. 2.

Developmental patterns of 2-AG biosynthetic enzymes. Western blot analysis of DAGL-α and β protein expression in neocortical total extracts. Ponceau S Stain confirmed equal loading. The representative blot shows the developmental patterns of DAGL-α and β protein expression (n = 8 pups/group; *p < 0.05 compared with the P2 group). Statistical analysis was conducted using one-way ANOVA with Bonferroni's post hoc tests. The error bars represent the SEM. HP, hippocampus; NC, neocortex.

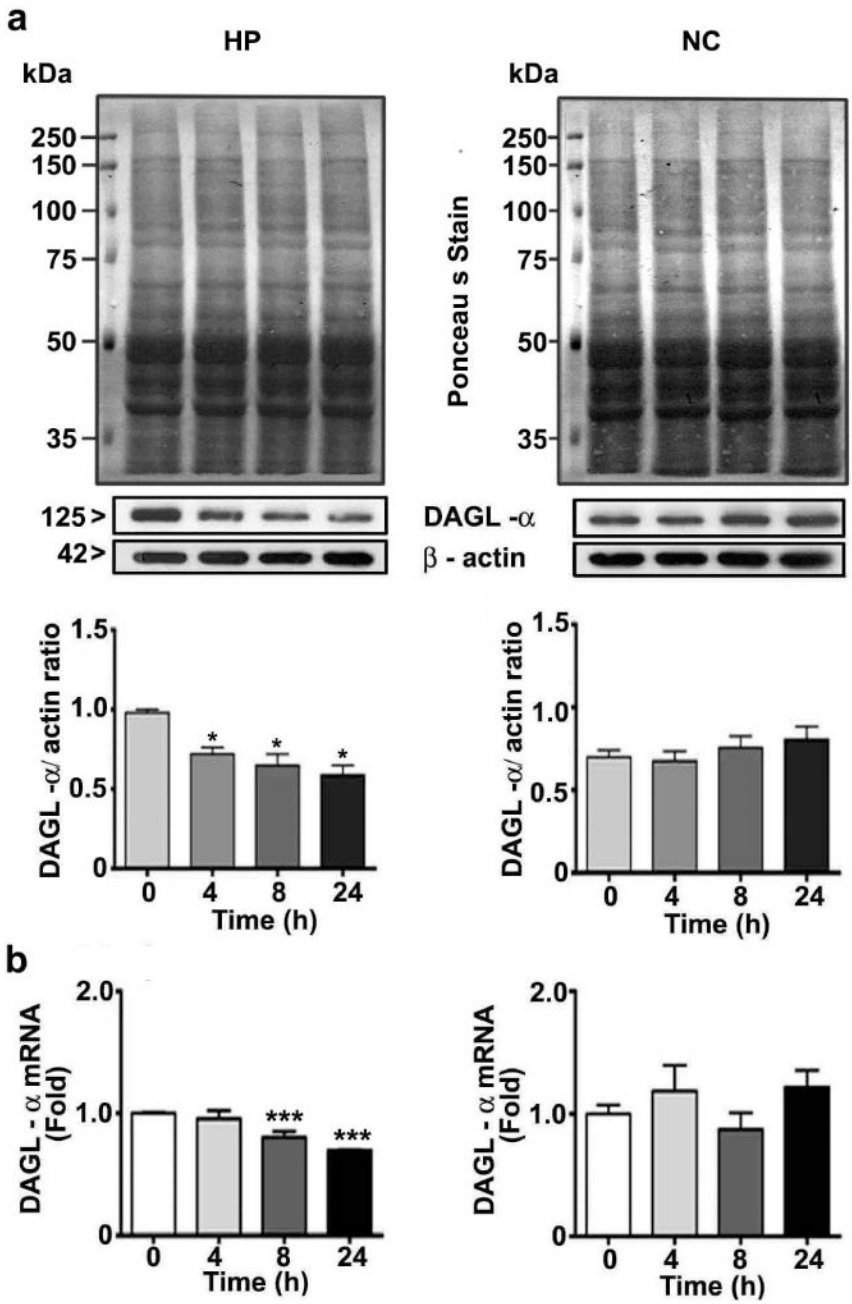

Fig. 3.

The ethanol treatment of P7 mice reduces the DAGL-α level. **(a)** DAGL-α levels in hippocampal and neocortical cytosolic extracts were determined by Western blot analysis. Blots were stained with Ponceau S to confirm equal loading in each lane, and β-actin was used as a loading control (n = 15 pups/group). (**b**) DAGL-α mRNA levels in hippocampal and cortical total extracts from the saline- and ethanol-treated groups (n = 15 pups/group) were measured by qPCR (*$p < 0.05$; ***$p < 0.01$). Statistical analysis was conducted with one-way ANOVA with Bonferroni's post hoc tests. The error bars represent the SEM. HP, hippocampus; NC, neocortex.

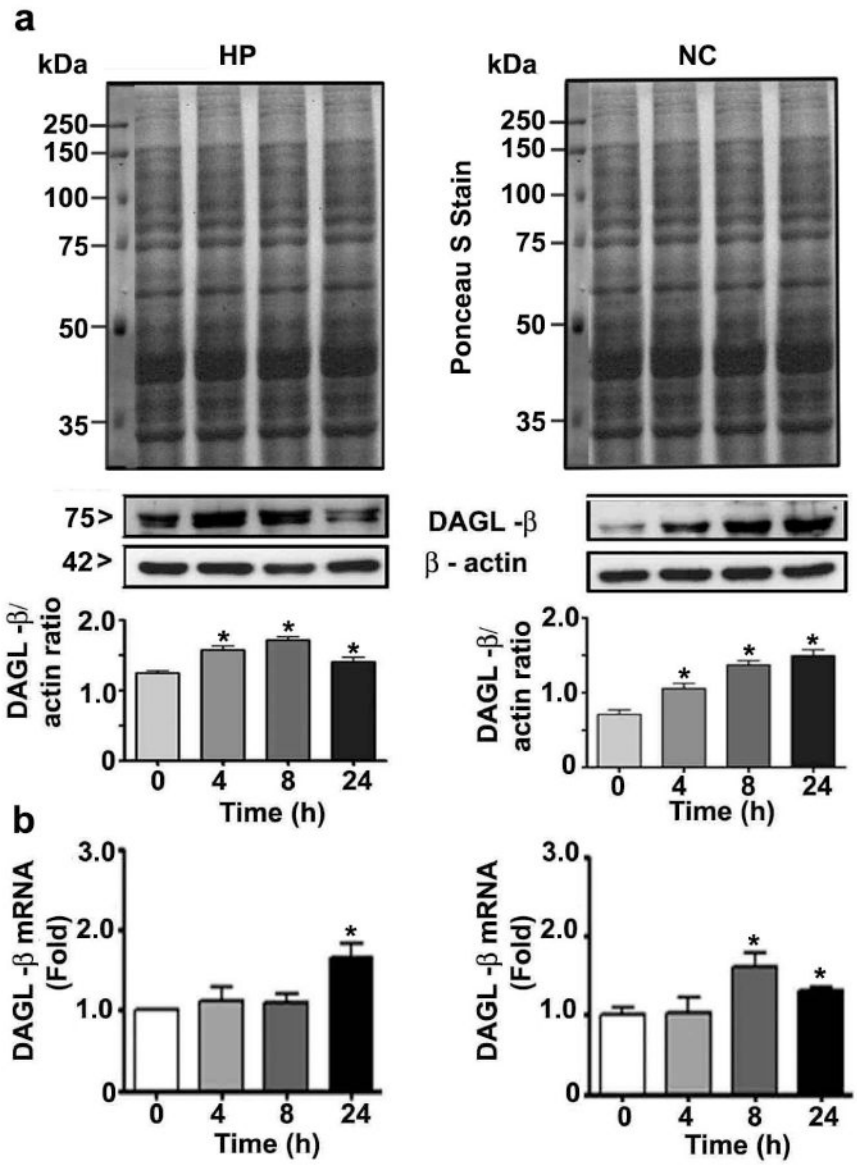

Fig. 4.

DAGL-β levels were enhanced in P7 mice treated with ethanol. **(a)** Hippocampal and neocortical levels of DAGL-β were determined in cytosolic extracts by Western blot analysis. Blots were stained with Ponceau S to confirm equal loading in each lane, and β-actin was used as a loading control (n = 15 pups/group). (**b**) qPCR was used to determine DAGL-β mRNA levels in hippocampal and cortical total extracts from the saline- and ethanol-treated groups (n = 15 pups/group; *p < 0.05). Statistical analysis was conducted using one-way ANOVA with Bonferroni's post hoc tests. The error bars represent the SEM. HP, hippocampus; NC, neocortex.

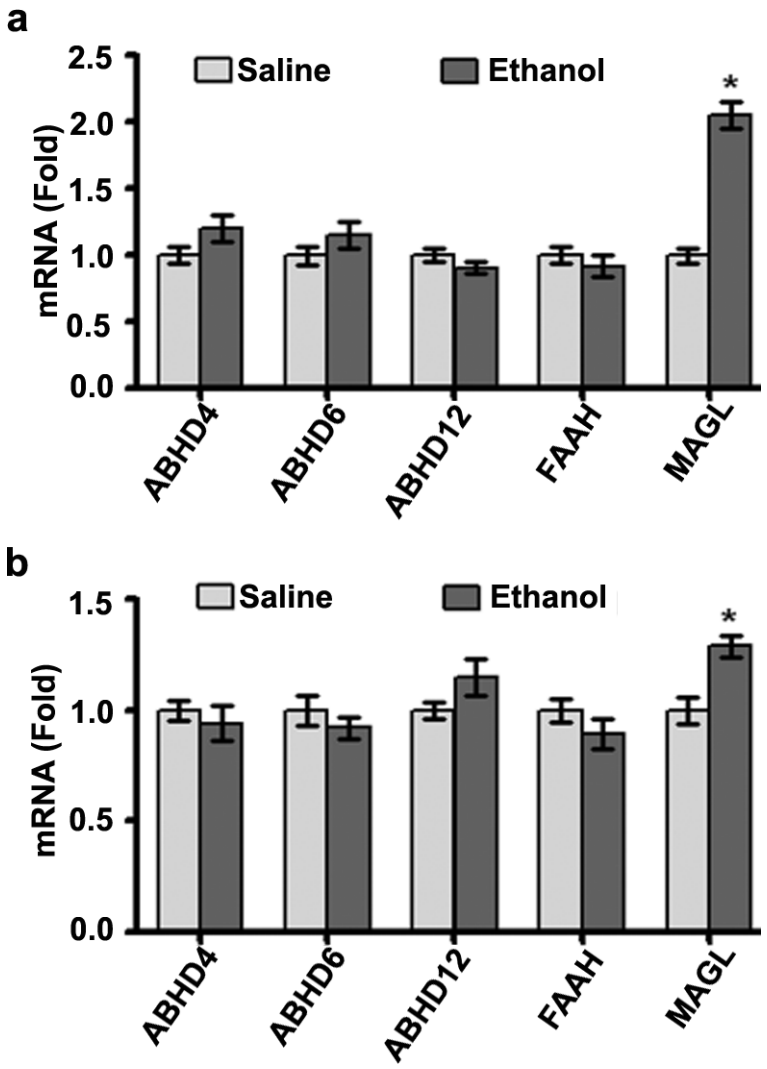

Fig. 5.

Influence of ethanol treatment of P7 mice on the mRNA levels of endocannabinoid-metabolizing enzymes. The mRNA levels of FAAH, ABHD4, ABHD6, ABHD12 and MAGL were determined in (**a**) hippocampal and (**b**) neocortex total extracts from the saline- and ethanol-treated groups (at 8 h after the first dose of ethanol or saline) by qPCR (n = 10 pups/group; *$p < 0.05$). Statistical analysis was conducted using one-way ANOVA with Bonferroni's post hoc tests. The error bars represent the SEM. HP, hippocampus; NC, neocortex.

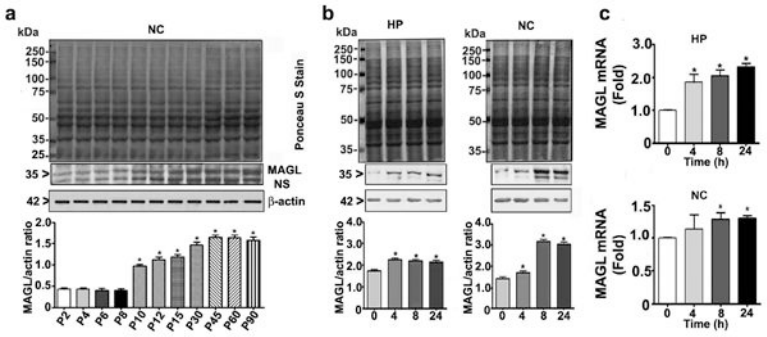

Fig. 6.

Developmental pattern and influence of ethanol treatment of P7 mice on MAGL expression. (**a**) Western blot analysis of MAGL expression in neocortical total extracts. Blots were stained with Ponceaus s Stain to confirm equal loading. The representative blot shows the developmental patterns of MAGL protein expression (n = 8 pups/group; *p < 0.05 compared with the P2 group). (**b**) Western blot analysis of MAGL in hippocampal and neocortex extracts from the saline and ethanol-treated groups (*p < 0.05). (**c**) MAGL mRNA levels in hippocampal and cortical total extracts from the saline- and ethanol-treated groups were measured by qPCR (n = 8 pups/group compared with the 0.0 control group; *p < 0.05). Statistical analysis was conducted using one-way ANOVA with Bonferroni's post hoc tests. The error bars represent the SEM. HP, hippocampus; NC, neocortex.

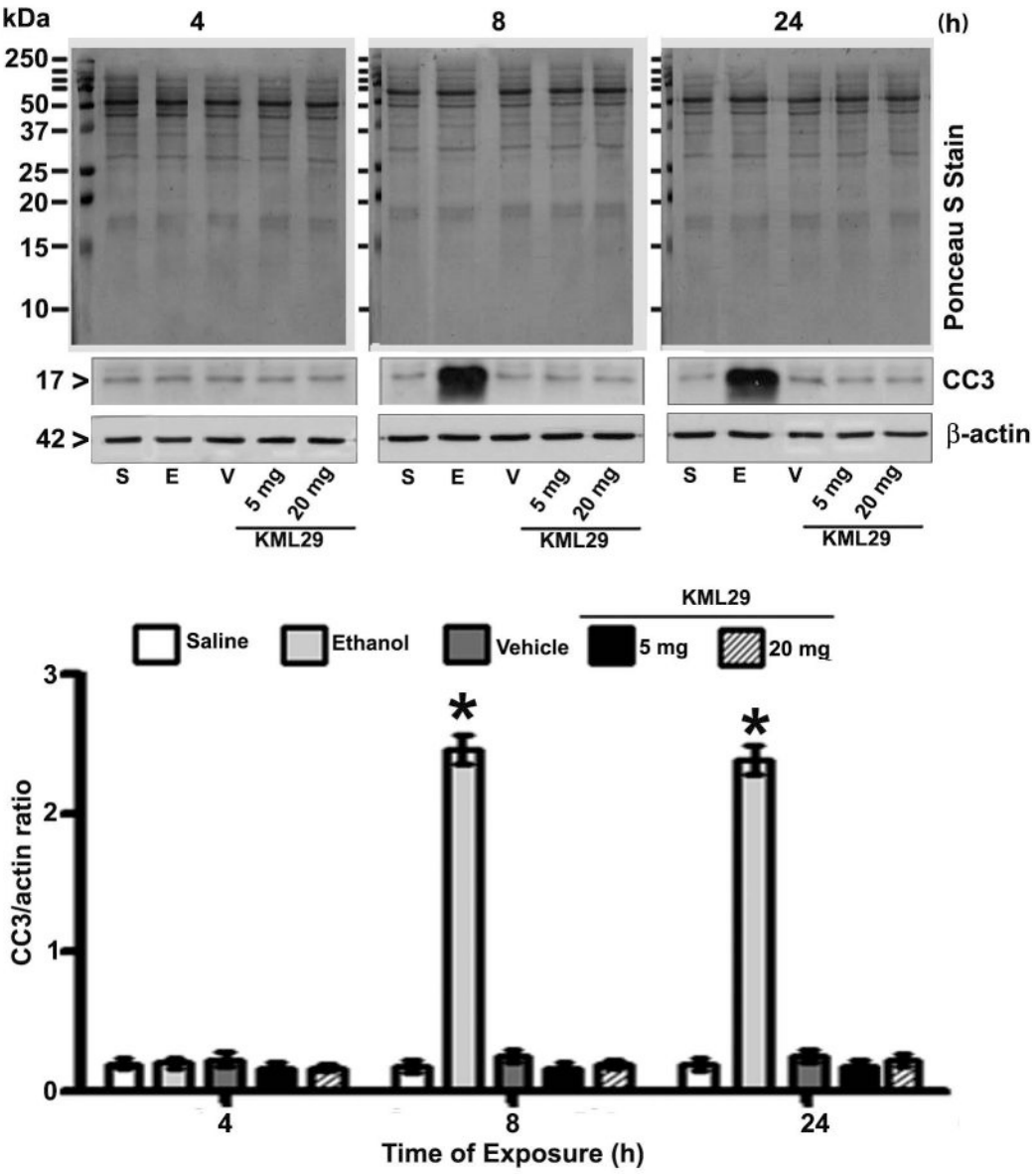

Fig. 7.

Pharmacological inhibition of MAGL by KML29 failed to induce caspase-3 activation in P7 mice. Mice were treated for 4-24 h with two doses of KML29 (5 and 20 mg/kg, s.c.) or vehicle and CC3 levels were determined in neocortex tissue by a Western blot analysis ($p > 0.05$). P7 were also treated with ethanol and used as positive controls. The error bars represent the SEM. S, saline; E, ethanol; V, vehicle.

Table 1

Ethanol-treatment of P7 mice enhances AEA but not 2-AG contents in hippocampus and neocortex tissues.

Endocannabinoids	Hippocampus		Neocortex	
	Saline	Ethanol	Saline	Ethanol
AEA (pmol/g)	6.54 ± 0.38	8.37 ± 0.17*	4.51 ± 0.11	6.87 ± 0.24*
2-AG (nmol/g)	32.84 ± 1.93	32.92 ± 1.63	31.67 ± 1.24	31.94 ± ` 0.58

AEA and 2-AG levels in hippocampal and neocortical total extracts were analyzed by LC-MS (n = 10 pups/group)

*($p < 0.05$ vs. saline-treated group).

All statistical analyses were conducted using one-way ANOVA with Bonferroni's post hoc tests. The error bars represent the SEM.