OXFORD

International Journal of Neuropsychopharmacology, 2015, 1–15

doi:10.1093/ijnp/pyu028
Research Article

RESEARCH ARTICLE

Ethanol Exposure Induces Neonatal Neurodegeneration by Enhancing CB1R Exon1 Histone H4K8 Acetylation and Up-regulating CB1R Function causing Neurobehavioral Abnormalities in Adult Mice

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Abstract

Background: Ethanol exposure to rodents during postnatal day 7 (P7), which is comparable to the third trimester of human pregnancy, induces long-term potentiation and memory deficits. However, the molecular mechanisms underlying these deficits are still poorly understood.
Methods: In the present study, we explored the potential role of epigenetic changes at cannabinoid type 1 (CB1R) exon1 and additional CB1R functions, which could promote memory deficits in animal models of fetal alcohol spectrum disorder.
Results: We found that ethanol treatment of P7 mice enhances acetylation of H4 on lysine 8 (H4K8ace) at CB1R exon1, CB1R binding as well as the CB1R agonist-stimulated GTPγS binding in the hippocampus and neocortex, two brain regions that are vulnerable to ethanol at P7 and are important for memory formation and storage, respectively. We also found that ethanol inhibits cyclic adenosine monophosphate response element-binding protein (CREB) phosphorylation and activity-regulated cytoskeleton-associated protein (Arc) expression in neonatal and adult mice. The blockade or genetic deletion of CB1Rs prior to ethanol treatment at P7 rescued CREB phosphorylation and Arc expression. CB1R knockout mice exhibited neither ethanol-induced neurodegeneration nor inhibition of CREB phosphorylation or Arc expression. However, both neonatal and adult mice did exhibit enhanced CREB phosphorylation and Arc protein expression. P7 ethanol-treated adult mice exhibited impaired spatial and social recognition memory, which were prevented by the pharmacological blockade or deletion of CB1Rs at P7.
Conclusions: Together, these findings suggest that P7 ethanol treatment induces CB1R expression through epigenetic modification of the CB1R gene, and that the enhanced CB1R function induces pCREB, Arc, spatial, and social memory deficits in adult mice.

Keywords: cannabinoid receptor system, epigenetics, FASD, memory loss, synaptic signaling

Received: May 23, 2014; **Revised:** August 13, 2014; **Accepted:** September 03, 2014

Introduction

Alcohol consumption during pregnancy exposes fetal brains to ethanol that causes various birth defects (Jones and Smith, 1973) in humans, collectively known as fetal alcohol spectrum disorders (FASDs; Streissguth et al., 1990). The consequential neurological abnormalities (Goodman et al., 1999; Mattson et al., 1999) are understood to be one of the major causes of intellectual disability in Western nations (Mattson et al., 2011). The studies using developmental animal models have long established that fetal ethanol exposure is associated with enormous reduction in the number of neurons in numerous brain regions, including the hippocampus (Olney, 2004), in addition to long-lasting synaptic and memory deficits in adult rodents (Izumi et al., 2005; Wilson et al., 2011; Sadrian et al., 2012; Subbanna et al., 2013a). Several pathways appear to activate neuronal death by ethanol; however, recent studies suggest that the endocannabinoid system not only contributes to neurodegeneration (Hansen et al., 2008; Subbanna et al., 2013a), but also plays a significant role in the development of synaptic and learning and memory deficits in adult mice (Subbanna et al., 2013a).

The endocannabinoid system includes endogenous ligands, cannabinoid type 1 (CB1R) and 2 receptors, and synthesizing and degrading enzymes (Piomelli, 2003; Basavarajappa, 2007; Subbanna et al., 2013a). A substantial amount of previous research has demonstrated multiple ways in which the endocannabinoid system regulates synaptic events (Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001; Bacci et al., 2004) in the developing (Berghuis et al., 2007; Mulder et al., 2008; Subbanna et al., 2013a) and adult brain (see Basavarajappa et al., 2009). Research findings from animal and human studies imply that the endocannabinoid system is one of the most relevant biochemical systems mediating the action of ethanol in multiple brain regions (Basavarajappa et al., 1998, 2003, 2006, 2008; Basavarajappa and Hungund, 1999a, 1999b; Roberto et al., 2010; DePoy et al., 2013; Hirvonen et al., 2013; Subbanna et al., 2013a; Ceccarini et al., 2014).

The CB1R is one of the most abundant inhibitory G-protein-coupled receptors expressed in the brain (Howlett et al., 1986; Herkenham et al., 1990) and is primarily located on presynaptic terminals, where it controls neurotransmitter release (Matyas et al., 2007). The ability of the CB1R to suppress neurotransmission allows endogenous cannabinoids such as anandamide (AEA) and 2-arachidonylglycerol to prevent the recruitment of new synapses (Kim and Thayer, 2001), which has a profound negative impact on neuronal communication, learning, and memory (Castellano et al., 2003; Mechoulam and Parker, 2013; Subbanna et al., 2013a). Moreover, cannabis use during brain development induces several specific human developmental disorders (Stefanis et al., 2004), including fetal alcohol syndrome–like deficits (Wu et al., 2011), which are likely mediated through the activation of CB1Rs.

Recently, epigenetic alterations have been shown to play a role in both normal development and several human developmental disorders (Campuzano et al., 1996; Petronis, 2003; Makedonski et al., 2005; Ryu et al., 2006; Warren, 2007; Gavin and Sharma, 2010), and have been implicated in developmental ethanol effects (Kaminen-Ahola et al., 2010; Bekdash et al., 2013; Perkins et al., 2013), including neurodegeneration (Subbanna et al., 2013b; Subbanna et al., 2014). Although the detailed mechanisms are not yet clear, we have recently shown that P7 ethanol treatment increases AEA/CB1R signaling, results in neonatal neurodegeneration, and contributes to the development of synaptic and object recognition memory deficits relevant to FASD (Subbanna et al., 2013a). In the present study, we explored the epigenetic and CB1R-mediated signaling events that may directly cause the neurodegeneration in neonatal mice and spatial and social recognition memory deficits in adult mice.

Methods

Animals and Treatment

Male C57BL/6J, CB1R wild type (WT), and knock out (KO) mice (Subbanna et al., 2013a) on a C57BL/6J background were generated from a heterozygous breeding colony at NKI. C57BL/6J, CB1RWT, and KO mice were housed in groups under standard laboratory conditions (12 h light/dark cycle) with food and water available *ad libitum*. Animal care and handling procedures followed institutional (NKI IACUC) and National Institutes of Health guidelines. Ethanol (2.5 g/kg s. c. at 0 h and again at 2 h) and [N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1 H-pyrazole-3-carboxamidehydrochloride)] (SR141716A) (SR; 1 mg/kg, 10 µl/g body weight) treatment and determination of ethanol levels were carried out as previously described by our laboratory (Subbanna et al., 2013a; Subbanna et al., 2013b). Three- to four-month-old mice were used for several analyses, as described below. For independent experiments, 5–8 animals were used.

ChIP Assay

Chromatin immunoprecipitation (ChIP) assay was performed as described before (Subbanna et al., 2014). For the ChIP assay, pups were sacrificed by decapitation and the hippocampus and neocortex were dissected 8 h after the first saline or ethanol injection. Tissue (25 mg) was fixed by 1% formaldehyde, homogenized, and subjected to DNA shearing; the amount of sample was normalized to contain equivalent protein amounts. Chromatin was immunoprecipitated with anti-acetyl histone H4K8 (# 07-328; Millipore) and anti-H3K9me2 (#4658; Cell Signaling) antibodies. As a negative control, samples were immunoprecipitated with rabbit IgG (Millipore). Immune-complexes were collected and processed as described before (Subbanna et al., 2014) using primers for mouse CNR1 (CB1R) exon I (mouse CNR1 219 F 5'-AGGAGACACAACCAACATTACA-3', mouse CNR1 277 P 5'-AACGAGGACAACATCCAGTGTGGG-3', and mouse CNR1 307 5'-TGAAGCACTCCATGTCCATAAA-3'). Relative quantification for acetylated and methylated histone-associated genes in saline and ethanol groups was calculated by the ΔΔCt method (Schmittgen and Livak, 2008).

Protein extraction, Electrophoresis, and Immunoblotting

For Western blot analysis, homogenates from the flash frozen hippocampus (HP) and neocortex (NC) from neonates and adult (P 90) was processed to prepare nuclear and total extracts (Basavarajappa et al., 2014; Basavarajappa and Subbanna, 2014) as described previously (Lubin and Sweatt, 2007; Subbanna et al., 2013b). The samples were prepared in a sample buffer as described previously (Basavarajappa et al., 2008; Subbanna et al., 2013a). Membranes were Ponceau S stained to confirm equal loading in each lane and were incubated in a primary antibody: anti-rabbit-pCREB (Ser133; # 9198, 1:1 000) and anti-rabbit-CREB (# 9197, 1:1 000; Cell Signaling Technology), anti-mouse activity-regulated cytoskeleton-associated protein (Arc; #sc 17839, 1:1 000; Santa Cruz Biotechnology), anti-rabbit cleaved caspase-3

(CC3; # 9661, 1:1 000), or anti-mouse-β-actin (#3700, 1:1 000; Cell Signaling Technology) for 3 h at room temperature or overnight at 4°C and processed as described previously (Basavarajappa et al., 2008). Incubation of blots with a secondary antibody (goat anti-mouse peroxidase conjugate, #AP 124P, 1:5 000; goat anti-rabbit, #AP132P, 1:5000; Millipore) alone did not produce any bands.

CB1R-Binding Assay

The CB1R-binding assay was performed with [^{3}H] CP-55,940 as the labeled ligand. A rapid filtration–binding assay was followed by a procedure as described previously (Basavarajappa et al., 1998, 2006). HP and NC from P7 saline- and ethanol-treated mice were isolated and homogenized in an ice-cold homogenization buffer (Basavarajappa et al., 2014; Basavarajappa and Subbanna, 2014) containing a freshly added 1% protease inhibitor mixture (Roche). Plasma membranes (PMs) were prepared as described previously (Basavarajappa et al., 1998, 2006) and appropriate aliquots were stored at -80°C until use. Assay solutions were incubated in silicone-treated tubes for 60 min at 30°C, with a final assay volume of 0.5 ml containing 4 nM [^{3}H] CP-55,940 and a final PM concentration of 0.02 mg protein/ml. Nonspecific binding was determined in the presence of 30 μM CP-55,940 and was subtracted to yield specific binding values. Bound [^{3}H] CP-55,940 was harvested by vacuum filtration through Whatman GF/B filters with a Brandel Cell Harvester. Radioactivity was determined by liquid scintillation counting after overnight incubation of filters in 5 ml of scintillation mixture UniverSol.

Agonist-Stimulated [^{35}S] GTPγS Binding

CP-55,940-stimulated [^{35}S] GTPγS binding was performed as described previously (Basavarajappa and Hungund, 1999a; Basavarajappa et al., 2006). PMs from HP and NC from P7 saline- and ethanol-treated mice were diluted in an assay buffer. Homogenates (20 μg of protein) were incubated with 0.1 nM [^{35}S] GTPγS, appropriate concentrations of CP55,940 in an assay buffer containing 100 μM GDP, and 0.1% BSA in a final volume of 0.5 ml for 60 min at 30°C. Nonspecific binding was determined in the presence of 30 μM GTPγS and was subtracted to yield specific binding values. Bound [^{35}S] GTPγS was determined by liquid scintillation counting after overnight incubation of filters in 5 ml of UniverSol scintillation fluid.

Behavioral Testing

Spontaneous Alternation Y Maze Task

Spontaneous alternation (Dember and Fowler, 1958) was tested as described previously (Holcomb et al., 1998) using the symmetrical Y maze exactly as described previously (Basavarajappa et al., 2014; Basavarajappa and Subbanna, 2014). Briefly, this experiment included three- to four-month-old male C57BL/6J mice treated at P7 with saline, ethanol, SR, or ethanol + SR, as well as CB1RWT and KO mice treated with saline or ethanol (n = 8/group). Percentage alternation is the number of trials containing entries into all three arms divided by the maximum possible alternations (the total number of arms entered minus 2) x 100.

Spatial Recognition Memory Using the Y Maze

Spatial recognition memory (Dellu et al., 1992) was tested as described previously (Sarnyai et al., 2000) using the symmetrical Y maze exactly as described previously (Basavarajappa et al., 2014; Basavarajappa and Subbanna, 2014). Briefly, this experiment included three- to four-month-old male C57BL/6J mice treated at P7 with saline, ethanol, SR, or ethanol + SR as well as CB1RWT and KO mice treated with saline or ethanol (n = 8/group). The number of entries and the time spent in each arm, as well as the first choice of entry, were registered manually and from video recordings by an observer blind to the treatment or genotype of the mice.

SRM

A social recognition memory (SRM) test (Thor et al., 1982) was performed exactly as previously described (Subbanna and Basavarajappa, 2014). In brief, three- to four-month-old male C57BL/6J mice treated at P7 with saline, ethanol, SR, or ethanol + SR as well as CB1RWT and KO mice treated with saline or ethanol (n = 8/group) were used. Each mouse was placed into an individual cage in an observation room under dim light and was allowed to habituate to the new environment for 15 min immediately prior to the experimental sessions. A male juvenile mouse (3–4 weeks old) was placed into a cage with an adult for an initial interaction trial of 2 min. Behaviors that were scored as social investigation were previously described (Subbanna and Basavarajappa, 2014). The percentage of social investigation was calculated by dividing the investigation time during the second exposure by the initial investigation time × 100.

Statistics

A statistical comparison of the data was performed by either a student's *t*-test, one-way analysis of variance (ANOVA), or two-way ANOVA with Bonferroni's post hoc test. In all of the comparisons, $p < 0.05$ was considered to indicate statistical significance. The statistical analyses were performed using Prism software (GraphPad).

Results

Neonatal Exposure to Ethanol Enhances H4K8 Acetylation and Demethylates H3K9 at CB1R Exon1

We used ChIP assay to determine whether CB1R transcriptional activation involves specific epigenetic modification of histone proteins in exon1 of the CB1R gene. The results indicated that ethanol treatment increased acetylated H4K8 levels (Figure 1a and b; $p < 0.001$) and reduced dimethylated H3K9 (Figure 1c and d) at CB1R exon I in the HP ($p < 0.01$) and NC ($p < 0.05$), which is correlated with increased CB1R transcription (Subbanna et al., 2013a).

Enhanced CB1R Binding and CB1R Agonist-Stimulated GTPγS Binding During Ethanol-Induced Neurodegeneration in the Developing Brain

Administration of ethanol to mouse pups at P7 resulted in an ethanol level of ~0.47 ± 0.25 g/dL at 3 h that was gradually reduced to 0.27 ± 0.07 g/dL at 9 h following injection. This ethanol paradigm has been shown to produce a widespread pattern of neurodegeneration throughout the forebrain, including the HP and NC, as indicated by the formation of CC3 in ethanol-exposed brains.

Our current results demonstrated that P7 ethanol treatment significantly enhanced the specific binding of CP-55,940 in a time-dependent manner both in the HP and NC ($p < 0.05$; Figure 1e). Furthermore, to examine the function of CB1Rs after P7 ethanol treatment, we examined CP-55,940-stimulated

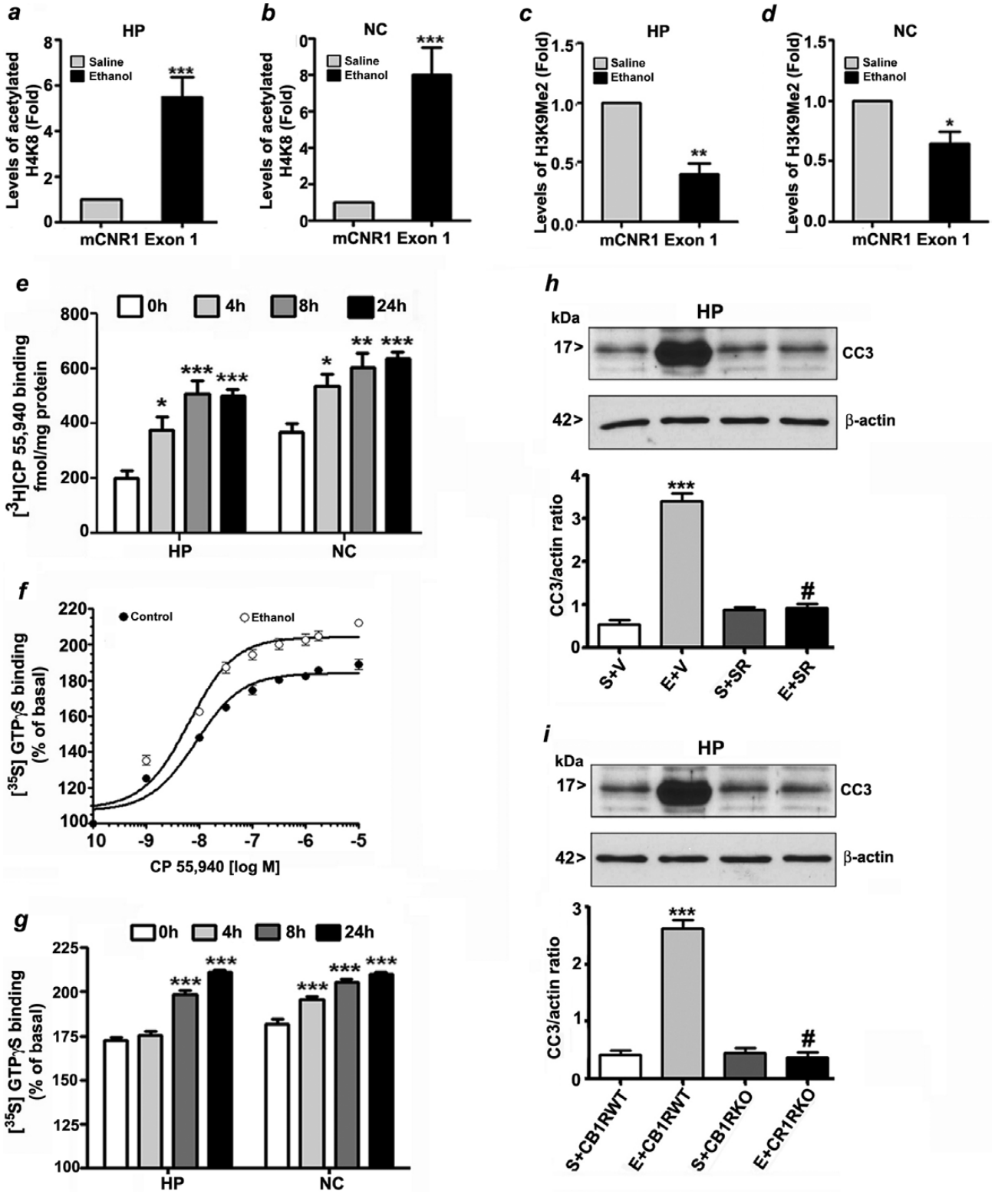

Figure 1. Enhanced H4K8 acetylation and reduced H3K9 dimethylation at exon1 of the CB1R gene regulate postnatal ethanol-induced expression of CB1R. ChIP analysis of the CB1R exon1 gene in hippocampal (HP) and neocortical (NC) tissues from the saline and ethanol groups (n = 8 pups/group) with anti-acetylated H4K8 (a and b) or anti-H3K9me2 (c and d) antibodies and levels of CB1R exon 1 chromatin enrichment in the IPs were measured by quantitative PCR. *$p < 0.05$,**$p < 0.01$,***$p < 0.001$; compared with respective saline group; student's *t*-test. Error bars = SEM. (e) CB1R binding was performed with PM (20 mg protein/ml) at 30°C for 60 min using [^{3}H] CP-55,940 as the labeled ligand and 30μM unlabeled CP-55,940 to define non-specific binding. Each point is the mean ± SEM (n = 12/group). [^{35}S] GTPγS binding was performed with various concentrations (f) or 2 μM (g) of CP-55, 940. Non-specific binding was determined in the presence of unlabeled GTPγS (30 μM). Data are expressed as the percentage of basal [^{35}S] GTPγS binding. Basal [^{35}S] GTPγS binding in the absence of CP-55,940 was ranged between 4.4 ± 0.50 (NC) and 5.0 ± 0.2 (HP) pmol/mg protein. Error bars = SEM (n = 12/group). (h) Mice pre-treated for 30 min with SR141716A (SR; 1 mg/kg) or vehicle were exposed to ethanol, and CC3 levels were determined by a Western blot analysis (***$p < 0.001$ vs. S+V; #$p < 0.001$ vs. E+V). (i) CB1R WT and KO mice were exposed to ethanol, and CC3 levels were determined by a Western blot analysis. β-actin was used as a loading control. Each point is the mean ± SEM. ***$p < 0.001$ vs S+CB1RWT; #$p < 0.001$ vs E+CB1RWT.

[^{35}S] GTPγS binding in PMs (Figure 1f). The increase in [^{35}S] GTPγS binding over basal levels stimulated by CP-55,940 was enhanced in a time-dependent manner in the HP and NC ($p < 0.05$) of the ethanol-treated groups compared to the saline groups (Figure 1g). To further evaluate the involvement of CB1R activity in ethanol-induced neurodegeneration, we used CB1RKO mice or a specific CB1R antagonist (SR) in WT mice and evaluated the ability of CB1R inhibition to prevent ethanol-induced CB1R-mediated neurodegeneration. The administration of SR (1 mg/kg) 30 min prior to ethanol treatment did not alter BALs (BAL peaked at 3 h at 0.47 ± 0.08 g/dL and was gradually reduced to 0.26 ± 0.07 g/dL at 9 h). In addition, the CB1RKO mice did not display altered BALs (BAL peaked at 3 h at 0.48 ± 0.09 g/dL and was gradually reduced to 0.28 ± 0.06 g/dL at 9 h). Two-way ANOVA analysis of CC3 levels suggested that the effects of ethanol (vs. saline $F_{1, 20} = 135$, $p < 0.001$) and SR (vs. saline, $F_{1, 20} = 69$, $p < 0.001$) were significant and that a significant interaction existed between ethanol and SR ($F_{1, 20} = 65$, $p < 0.001$; Figure 1h). Neither SR nor vehicle alone had any significant effects on CC3 levels in the absence of subsequent ethanol treatment. In addition, one-way ANOVA suggested that CB1RKO provided protection against P7 ethanol-induced neurodegeneration in the HP when compared with the WT mice (CC3: $F_{3, 20} = 220$, $p < 0.001$; Figure 1i). CB1RKO mice treated with saline showed normal CC3 levels, similar to observations in WT mice treated with SR alone.

Phosphorylation of CREB was Inhibited After Ethanol Exposure in Neonatal Mice

Our previous studies demonstrate that P7 ethanol treatment significantly reduces ERK1/2 phosphorylation (Subbanna et al., 2013a). To further assess the contribution of intracellular signaling events to the action of ethanol on the developing brain, we determined the levels of CREB phosphorylation by Western blot analysis using specific phospho-CREB antibodies. P7 ethanol treatment significantly reduced the pCREB but not the total CREB protein levels in the HP ($F_{3, 28} = 56$, $p < 0.001$) or the NC ($F_{3, 28} = 60$, $p < 0.001$; Figure 2a) at the 8 h and 24 h intervals.

Ethanol Exposure in Neonatal Mice Reduces Arc Protein Expression

To further assess the contribution of intracellular signaling events to the action of ethanol on the developing brain, we determined the levels of Arc by Western blot analysis using specific Arc antibodies. P7 ethanol treatment significantly reduced the Arc protein levels in the HP ($F_{3, 28} = 70$, $p < 0.001$) and NC ($F_{3, 28} = 55$, $p < 0.001$; Figure 2b) at 8 h and 24 h time points.

Impaired pCREB and Arc are Long-Lasting to Adulthood and Neuroprotective Effects of CB1R Blockade Involves pCREB and Arc Pathway

To elucidate the downstream intracellular pathways involved in the protective effects of the CB1R blockade, we studied the involvement of the pCREB and Arc pathway, a key regulator of cell survival (Luikart et al., 2008) and synaptic plasticity (Caroni et al., 2012). We investigated whether pre-treatment of SR, which prevents ethanol-induced neurodegeneration, could rescue these ethanol-induced pCREB and Arc deficits. Our results suggest that CREB phosphorylation, as well as

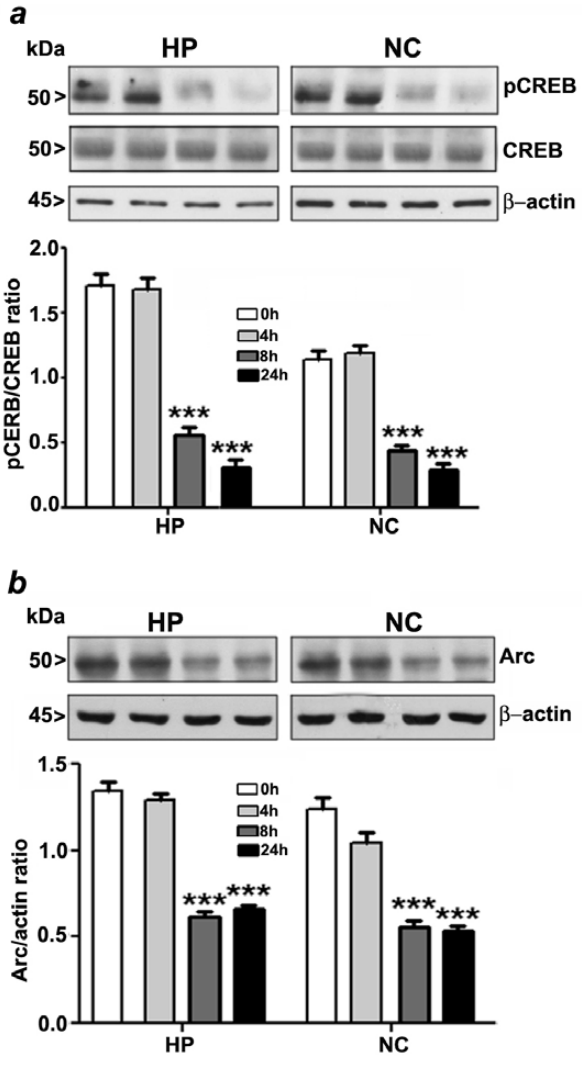

Figure 2. Ethanol inhibits CREB phosphorylation and Arc protein expression in the neonatal brain. (a) A Western blot analysis of pCREB and CREB in hippocampal and cortical nuclear extracts from the saline or ethanol-treated groups (***$p < 0.001$ vs. 0 h). (b) A Western blot analysis of Arc protein levels in hippocampal and neocortical total extracts from the saline- or ethanol-treated groups. β-actin was used as a loading control (n = 10 pups/group; ***$p < 0.001$ vs. 0 h). Statistical analysis was done using one-way ANOVA with Bonferroni's post hoc tests. Each point is the mean ± SEM. HP, hippocampus; NC, neocortex.

Arc protein expression, were rescued by SR pre-treatment (compared with the ethanol group) in neonatal (HP: pCREB, $F_{3, 20} = 55$, $p > 0.01$, Arc, $F_{3, 20} = 30$, $p > 0.01$; NC: pCREB, $F_{3, 20} = 35$, $p > 0.01$, Arc, $F_{3, 20} = 45$, $p > 0.01$, two-way ANOVA; Figure 3a and 4a) and adult mice tissues (HP: pCREB, $F_{3, 20} = 40$, $p > 0.01$, Arc, $F_{3, 20} = 25$, $p > 0.01$; NC: pCREB, $F_{3, 20} = 45$, $p > 0.01$, Arc, $F_{3, 20} = 55$, $p > 0.01$, two-way ANOVA; Figure 5a and 6a). We found that the total CREB protein levels were not altered in the ethanol-treated samples compared with the saline samples. In addition, SR did not alter the CREB protein levels in either the ethanol or saline samples of neonatal (Figure 3a) and adult (Figure 5a) rats. Similarly, CB1RKO mice, which do not exhibit ethanol-induced neurodegeneration, provided protection against P7 ethanol-induced inhibition of CREB phosphorylation and Arc expression in the neonatal (HP: pCREB, $F_{3, 20} = 42$, $p < 0.001$, Arc, $F_{3, 20} = 52$, $p < 0.001$; NC: pCREB, $F_{3, 20} = 58$, $p < 0.001$, Arc, $F_{3, 20} = 62$, $p < 0.001$, one-way ANOVA; Figure 3b and 4b) and

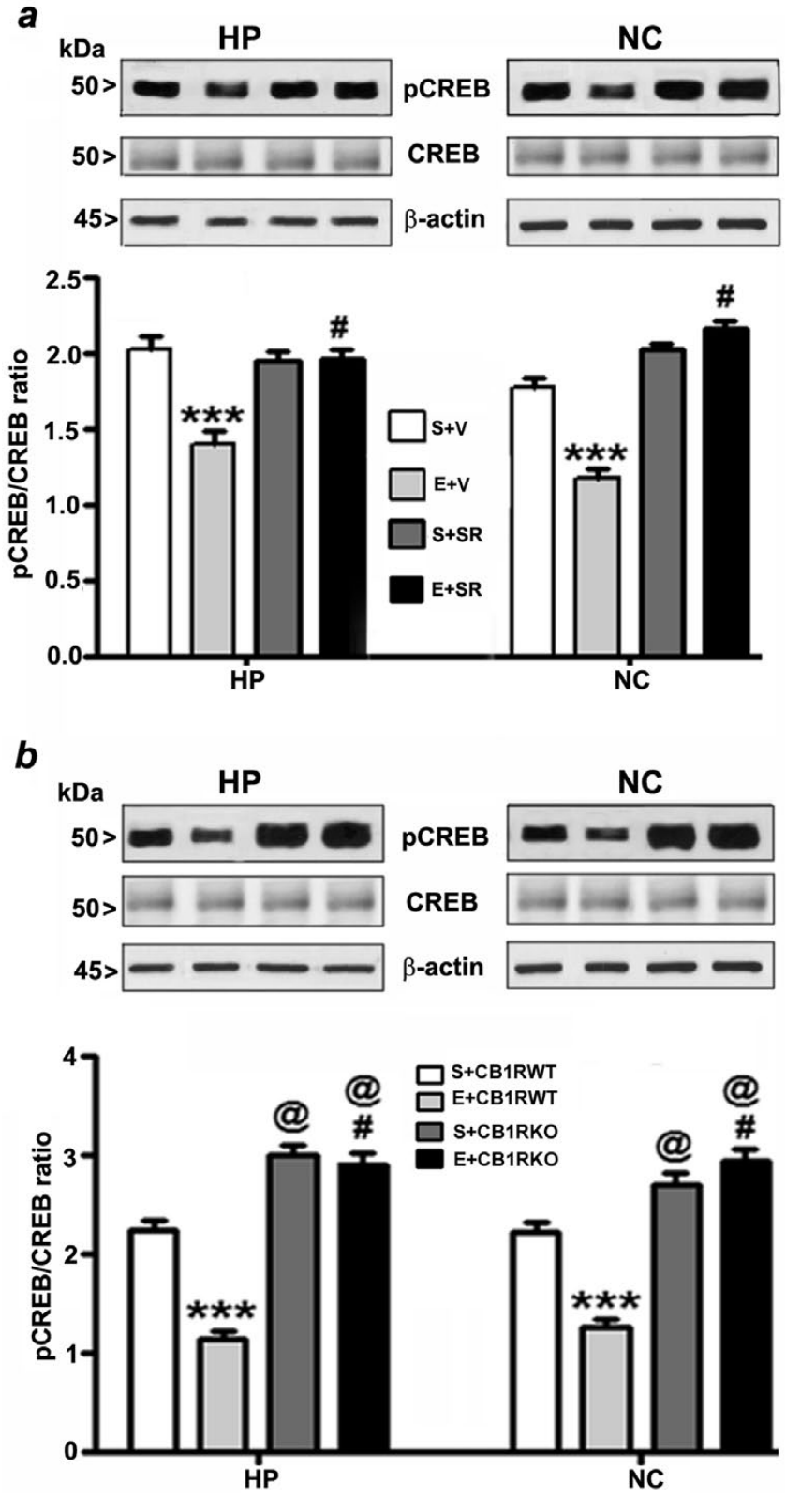

Figure 3. Pharmacological blockade or genetic ablation of CB1Rs provides protection against ethanol-induced inhibition of CREB phosphorylation in the neonatal mouse brain. (a) Hippocampal and neocortical nuclear extracts from the four treatment groups (S+V, E+V, S+SR, and E+ SR) were subjected to Western blot to analyze the levels of pCREB and CREB (n = 10 pups/group; ***$p < 0.001$ vs. S+V; #$p < 0.001$ vs. E+V). (b) Additional Western blot analyses were performed to determine the levels of pCREB and CREB in the hippocampal and cortical nuclear extracts obtained from the saline and ethanol-treated P7 CB1RWT and KO mice. The representative blots are shown for the hippocampal and cortical nuclear extracts (n = 10 pups/group; ***$p < 0.001$ vs. S+CB1RWT; #$p < 0.001$ vs. E+ CB1RWT; @$p < 0.001$ vs. S+CB1RWT). β-actin was used as a loading control. Two-way ANOVA with Bonferroni's post hoc tests was used for statistical analysis. Each point is the mean ± SEM. HP, hippocampus; NC, neocortex.

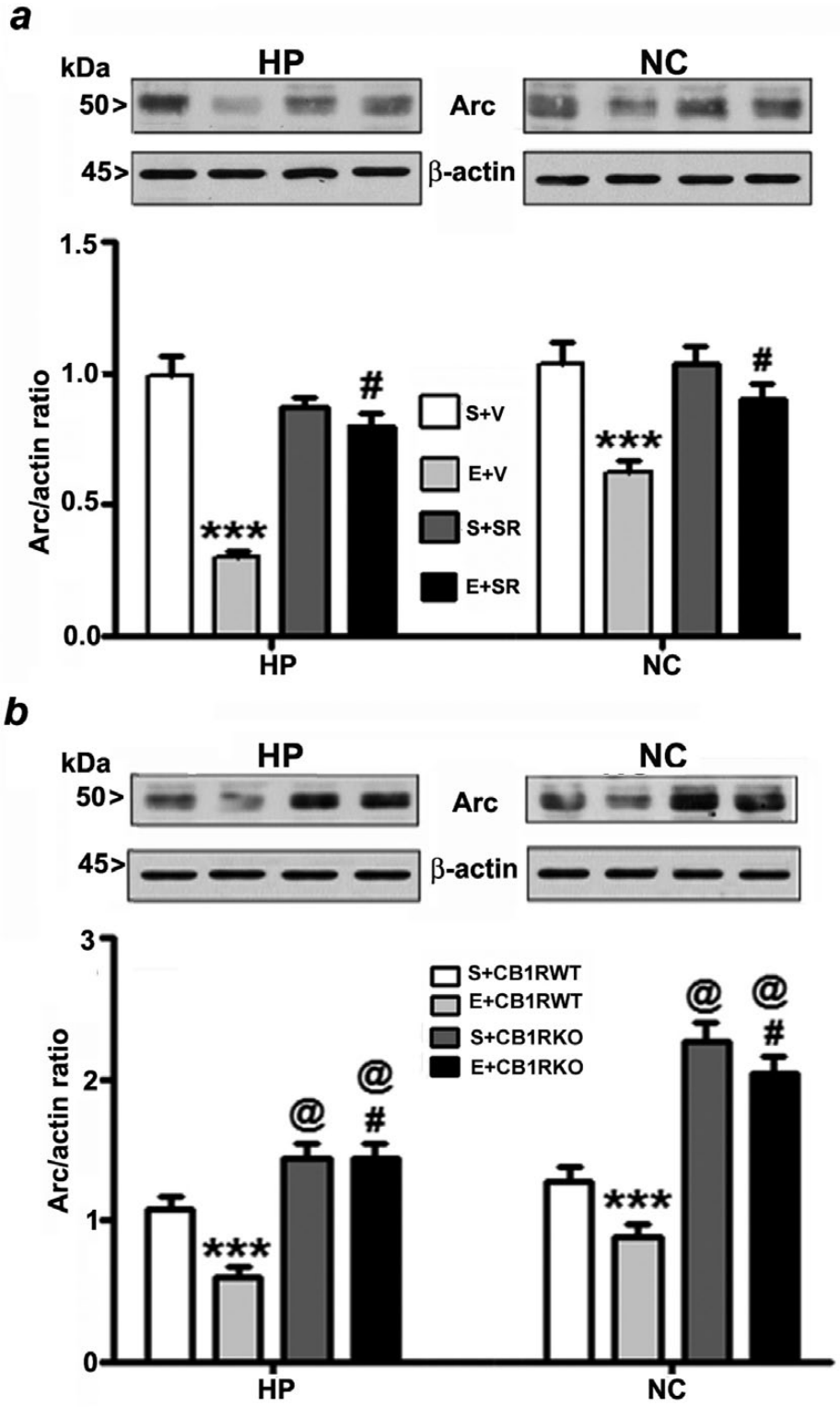

Figure 4. Pharmacological inhibition or genetic deletion of CB1Rs provides protection against ethanol-induced inhibition of Arc expression in the neonatal mouse brain. (a) Hippocampal and neocortical total extracts from the four treatment groups (S+V, E+V, S+SR, and E+ SR) were processed for Western blot to analyze the levels of Arc protein (n = 10 pups/group; ***$p < 0.001$ vs. S+V; #$p < 0.001$ vs. E+V). (b) To determine the Arc protein levels in saline and ethanol-treated CB1RWT and KO P7 mice samples, the hippocampal and neocortical total extracts were subjected to Western blot analyses. The representative blots are shown for the hippocampal and cortical cytosolic extracts (n = 10 pups/group; ***$p < 0.001$ vs. S+CB1RWT; #$p < 0.001$ vs. E+CB1RWT; @$p < 0.001$ vs. S+CB1RWT). Two-way ANOVA with Bonferroni's post hoc tests was used for statistical analysis. Each point is the mean ± SEM. HP, hippocampus; NC, neocortex.

adult mice tissues (HP: pCREB, $F_{3, 20} = 22$, $p < 0.01$, Arc, $F_{3, 20} = 32$, $p < 0.01$; NC: pCREB, $F_{3, 20} = 38$, $p < 0.01$, Arc, $F_{3, 20} = 32$, $p < 0.01$, one-way ANOVA; Figure 5b and 6b). In addition, neonatal and adult CB1RKO mice also exhibited enhanced CREB phosphorylation and Arc levels compared to their WT littermates ($p < 0.01$; Figure 5b and 6b).

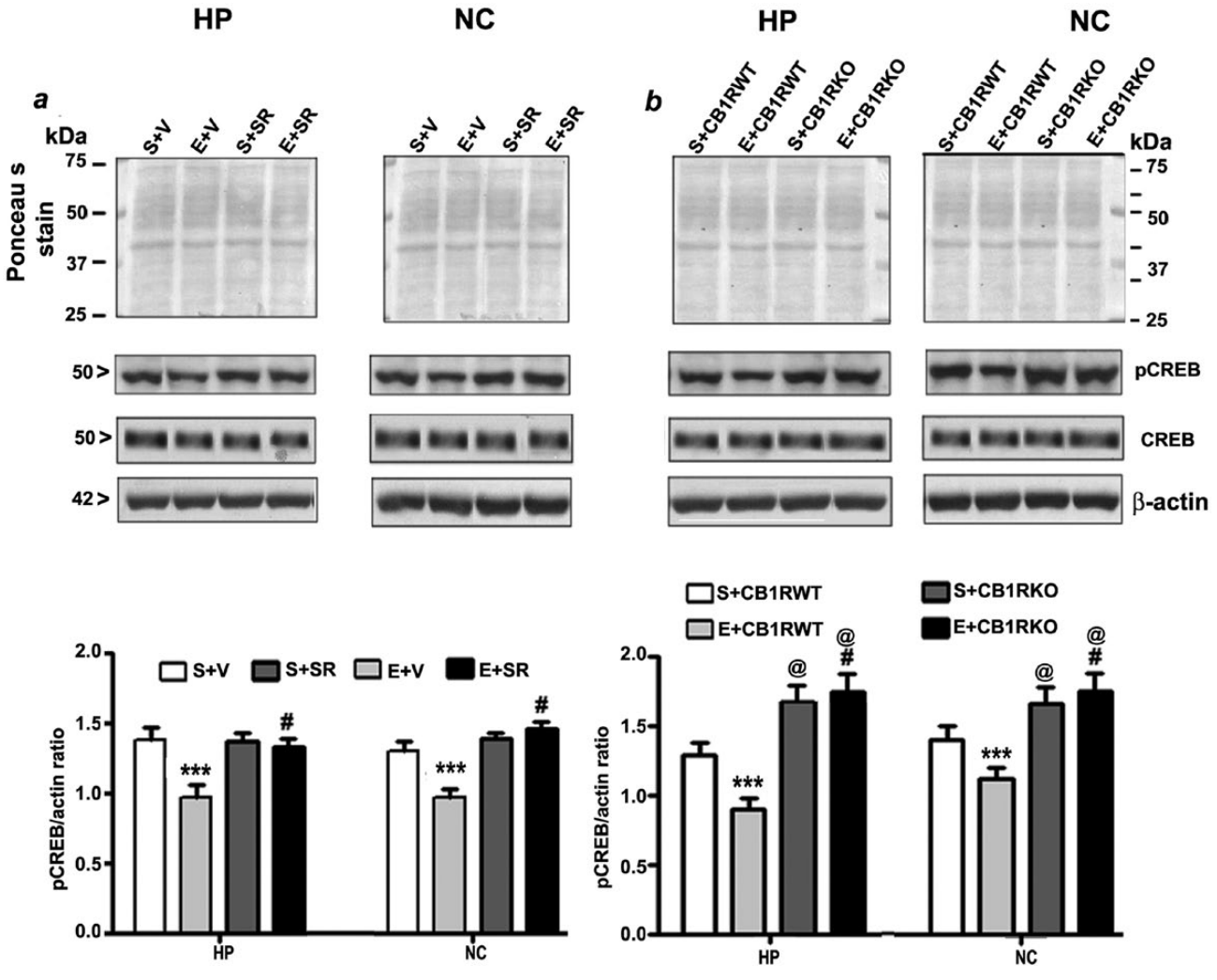

Figure 5. Pre-administration of SR141716A or genetic ablation of CB1Rs provides protection against P7 ethanol-induced inhibition of CREB phosphorylation in the adult mouse brain. (a) Hippocampal and neocortical nuclear extracts from the four treatment groups (S+V, E+V, S+SR and E+ SR) were subjected to Western blot to analyze the levels of pCREB and CREB (n = 8 mice/group; ***$p < 0.001$ vs. S+V; #$p < 0.001$ vs. E+V). (b) Additional Western blot analyses were performed to determine the levels of pCREB and CREB in the hippocampal and cortical nuclear extracts obtained from the P7 saline and ethanol-treated adult CB1RWT and KO mice. The representative blots are shown for the hippocampal and cortical nuclear extracts (n = 5 mice/group; ***$p < 0.001$ vs. S+CB1RWT; #$p < 0.001$ vs. E+ CB1RWT; @$p < 0.001$ vs. S+CB1RWT). Ponceau S staining and β-actin was used as a loading control. Two-way ANOVA with Bonferroni's post hoc tests was used for statistical analysis. Each point is the mean ± SEM. HP, hippocampus; NC, neocortex.

Pharmacological Blockade or Genetic Deletion of CB1Rs Before P7 Ethanol Treatment Rescues Memory Loss in Adult Mice

In our first behavioral test, adult mice treated with saline, ethanol (with vehicle), SR, or ethanol + SR at P7 were tested using spontaneous alternation in the Y maze. P7 ethanol, SR, or ethanol + SR treatment had no significant effect on exploratory activities assessed by the number of arm entries (Figure 7a) and time spent (Figure 7b) in each arm during Y-maze testing. Two-way ANOVA revealed that the ethanol-treated mice exhibited significantly reduced spontaneous alternation performance compared to saline-treated mice and that SR rescued these deficits ($F_{3,21} = 10$, $p < 0.001$; Figure 7c). Treatment with SR alone at P7 had no significant effect on spontaneous alternation performance. P7 ethanol treatment had no significant effect on the number of arm entries (Figure 7d) or the time spent in each arm (Figure 7e; exploratory activity) in either CB1RWT or KO mice. CB1RKO mice exhibited significantly enhanced spontaneous alternation behavior ($p < 0.01$) compared to WT mice. Notably, ethanol treatment at P7 failed to induce a spatial working memory deficit in the Y-maze test in adult CB1RKO mice ($p > 0.05$; Figure 7f).

In our second behavioral test, we examined spatial recognition memory using the Y-maze. Two-way ANOVA revealed that saline- and SR-treated mice entered more frequently into (Arm Entry: 1 h, $F_{3,21} = 21$, $p < 0.01$; 24 h, $F_{3,21} = 26$, $p < 0.01$) and spent more time in (Dwell Time: 1 h, $F_{3,21} = 61$, $p < 0.01$; 24 h, $F_{3,21} = 22$, $p < 0.01$) the novel, previously unvisited arm of the maze. In contrast, P7 ethanol-treated mice showed a reduced preference toward the novel arm ($p < 0.01$) and spent less time (Dwell Time: $p < 0.01$) in the novel arm compared to P7 saline-treated mice in both the 1 h (Figure 8a and b) and 24 h (Figure 8c and d) retention trials. SR pre-treatment rescued ethanol-induced impairments in the preference for the novel arm ($p < 0.01$) and time spent ($p < 0.01$) in the novel arm in both the 1 and 24 h retention trials. Although all saline- and SR-treated mice (combined 1 and 24 h) selected the novel arm as the first choice, ethanol-treated animals showed a reduced preference for the novel arm (Figure 8e),

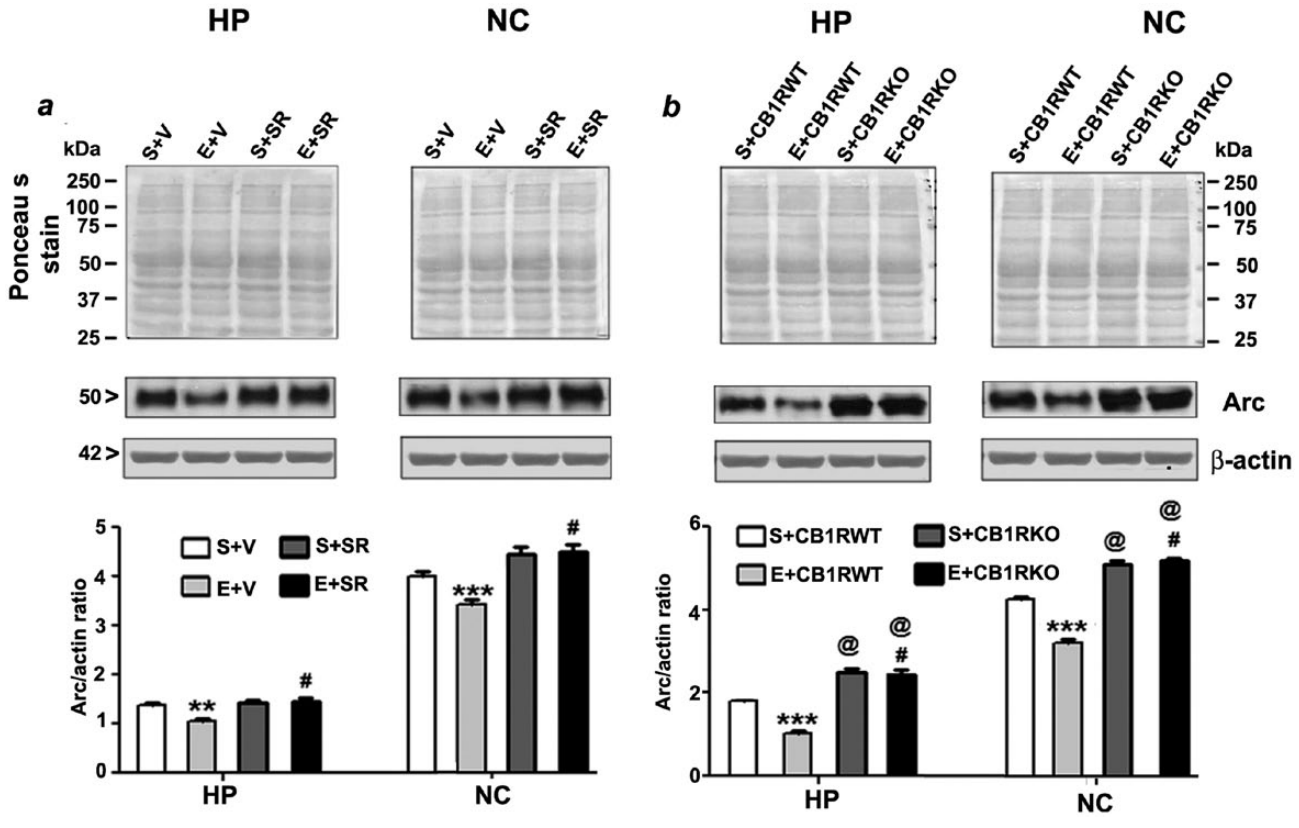

Figure 6. Pre-administration of SR141716A or genetic deletion of CB1Rs provides protection against ethanol-induced inhibition of Arc expression in the adult mouse brain. (a) Hippocampal and neocortical total extracts from the four treatment groups (S+V, E+V, S+SR, and E+ SR) were processed for Western blot to analyze the levels of Arc protein (n = 8 mice/group; $^{**}p < 0.01$; $^{***}p < 0.001$ vs. S+V; $\#p < 0.001$ vs. E+V). (b) To determine the Arc protein levels in P7 saline and ethanol-treated adult CB1RWT and KO mice samples, the hippocampal and neocortical total extracts were subjected to Western blot analyses. The representative blots are shown for the hippocampal and cortical cytosolic extracts (n = 5 mice/group; $^{***}p < 0.001$ vs. S+CB1RWT; $\#p < 0.001$ vs. E+CB1RWT; $@p < 0.001$ vs. S+CB1RWT). Ponceau S staining and β-actin was used as a loading control. Two-way ANOVA with Bonferroni's post hoc tests was used for statistical analysis. Each point is the mean ± SEM. HP, hippocampus; NC, neocortex.

which this was prevented by SR pretreatment ($F_{3,45} = 50$, $p < 0.01$). while CB1RKO mice showed an enhanced preference for the novel arm (Arm Entry, $p < 0.001$) and spent more time in the novel arm (Dwell Time, $p < 0.001$) compared to WT mice in both the 1 h (Figure 8f and g) and 24 h retention trials (Figure 8h and i). In addition, all saline- and ethanol-treated CB1RKO mice (combined 1 and 24 h) selected the novel arm as the first choice (Figure 8j).

The social investigation results revealed that ethanol-treated mice exhibited significantly-reduced short-term (Figure 9a) and long-term (Figure 9b) SRM performance compared to saline-treated mice. Two-way ANOVA revealed that SR pretreatment rescued ethanol-induced short-term ($F_{3,21} = 18$, $p < 0.01$) and long-term ($F_{3,21} = 14$, $p < 0.01$) SRM deficits compared to ethanol-treated mice. In addition, SR alone had no significant effects ($p > 0.05$) on SRM, and these mice exhibited normal SRM. Ethanol failed to impair SRM in CB1RKO mice, and KO mice exhibited normal SRM (Figure 9c and d).

Discussion

In this study, we demonstrate for the first time that transcriptional activation of CB1R followed by widespread neurodegeneration in the neonatal brain (Subbanna et al., 2013a) involves specific increases in H4K8 acetylation and demethylation of H3K9 at exon 1 in the CB1R gene. Dimethylation of histone H3K9 correlates with transcriptional silencing, whereas the acetylation of histone H4 at lysine 8 (H4K8ace) is linked to active transcription (Jenuwein and Allis, 2001). Our findings are consistent with postnatal ethanol-induced enhancement of active transcription of G9a gene (Subbanna et al., 2014). Demethylation of H3K9 found at the CB1R gene may be due to the global loss of the H3K9me2 mark secondary to postnatal-ethanol-induced caspase-3 mediated H3K9me2 degradation (Subbanna et al., 2013b). While studies related to epigenetic changes at the CB1R gene are either primitive (Wang et al., 2008) or have not yet been conducted, our unprecedented initial studies suggest that the epigenetic mark which regulates active gene transcription (Subbanna et al., 2013a; Subbanna et al., 2013b; Subbanna et al., 2014) also regulates the CB1R gene expression (Subbanna et al., 2013a) triggered by neonatal ethanol neurotoxicity. Ethanol-induced CB1R protein expression (Subbanna et al., 2013a) also reflected increases in CB1R levels in the PM preparations as shown by specific binding of CP55,940. In addition, our studies suggest that ethanol-enhanced CB1R proteins are functionally active at the PM because CP55,940-stimulated GTPγS binding also enhanced in parallel with CB1R levels. Although the studies on the consequences of enhanced CB1R function in neonatal mice are limited (Hansen et al., 2008; Subbanna et al., 2013a), our previous studies suggest that postnatal ethanol-enhanced CB1R function induces neurodegeneration in neonatal mice that leads to long-lasting deficits in long-term potentiation and object recognition test (ORT) in adult mice (Subbanna et al., 2013a).

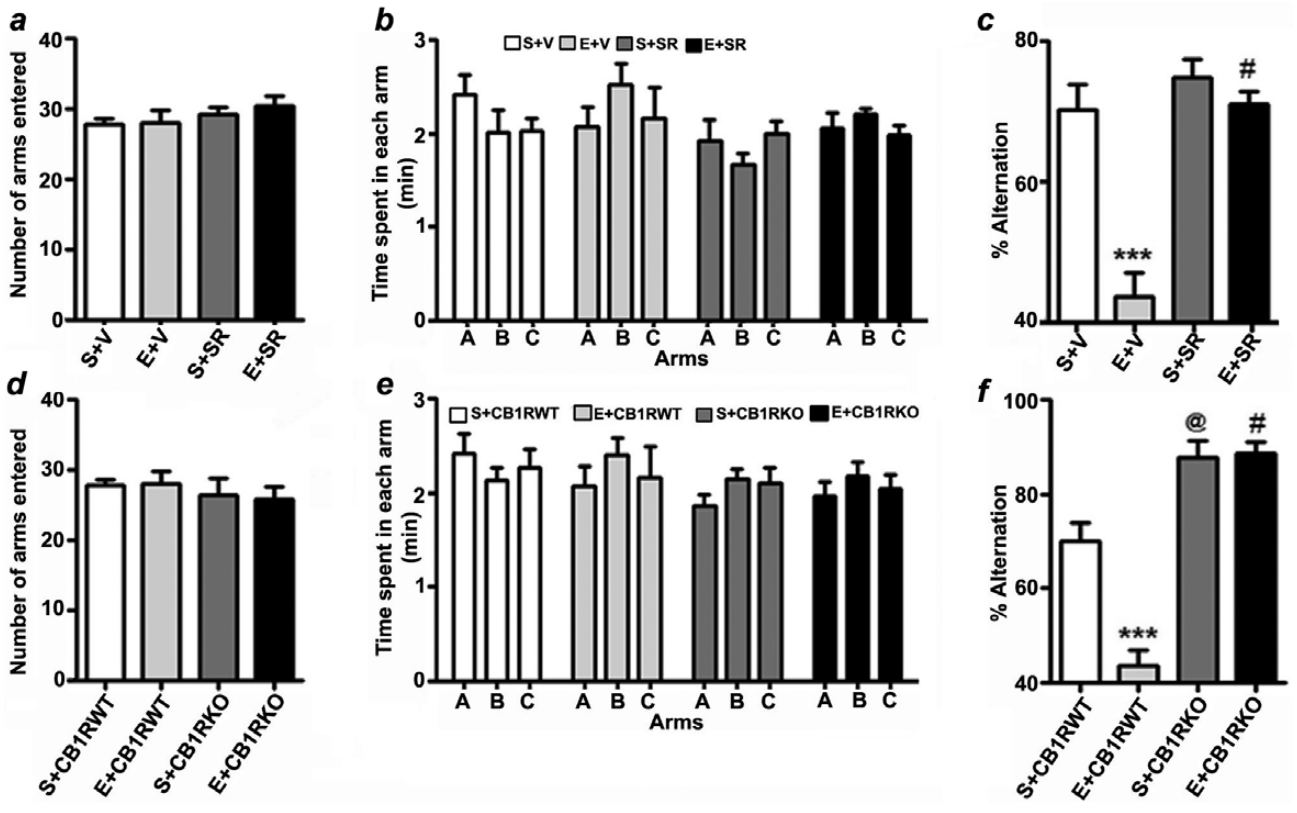

Figure 7. Prior blockade or genetic deletion of CB1Rs prevents P7 ethanol-induced spontaneous alternation performance deficits in adult mice. (a) Total number of arm entries reflecting exploratory activities of mice in the Y-maze does not differ between S+V, E+V, S+SR, and E+SR-treated mice. (b) The time spent in each arm was not different between the four groups ($p > 0.05$). (c) Spatial working memory of S+V, E+V, S+SR, and E+SR-treated mice were tested by spontaneous alternation performance in the Y-maze. Note that E+V-treated mice perform poorly compared to S+V-treated controls (***$p < 0.001$), while E+SR treatment restores E+V levels of alternation performance (#$p < 0.001$ versus E+V). n = 8 mice per group. One-way ANOVA with Bonferroni's post hoc test was used to analyze significant differences. (d) Total number of arm entries reflecting exploratory activities of mice in the Y-maze does not differ between the CB1RWT and KO with or without ethanol. (e) The time spent in each arm was not different between S+CB1RWT, E+CB1RWT, S+CB1RKO, and E+CB1RKO treated mice ($p > 0.05$). (f) Spontaneous alternation performance of CB1RWT and CB1RKO mice treated with or without saline or ethanol were tested in the Y-maze. Note that the performance of P7 saline-treated CB1RKO mice was significantly enhanced compared to P7 saline-treated CB1RWT mice (@$p < 0.001$), while ethanol failed to impair alternation performance in CB1RKO mice (#$p < 0.001$ vs. E + CB1RWT). n = 8 mice per group. One-way ANOVA with Bonferroni's post hoc test was used to analyze significant differences. Each point is the mean ± SEM.

Consistent with this observation, elevation of endogenous AEA through inhibition of fatty acid amide hydrolase during postnatal development, as observed with postnatal ethanol (Subbanna et al., 2013a), leads to impaired working memory in adult mice (Wu et al., 2014). CB1R has been shown to regulate the generation and maturation of excitatory and inhibitory neurons during brain development (Keimpema et al., 2013) and has also been shown to inhibit the release of glutamate and gamma aminobutyric acid (GABA) in matured neurons (Wilson and Nicoll, 2002). However, in neonatal mice, it appears that the ethanol blocking action at NMDARs and its stimulatory action at $GABA_A$Rs are mainly responsible for its neurodegenerative responses (Ikonomidou et al., 2000). While more research is warranted to understand the specific effects of ethanol-activated CB1R on NMDA- and GABA-mediated neurotransmission in neonatal mice, it appears that GABA is excitatory during the early stages of brain development and becomes inhibitory later on as adulthood approaches (Ben-Ari, 2002). Therefore, dysregulation of the CB1R pathway which regulates NMDA and GABA neurotransmission may have long-lasting consequences on synaptic function.

Our previous studies suggested the presence of a remarkable specificity involving the AEA/CB1R/ERK pathway, but not the AKT pathway (Young et al., 2008), in the regulation of ethanol-induced neonatal neurodegeneration (Subbanna et al., 2013a). Our current findings suggest that reduced CREB phosphorylation could be rescued by blockade or genetic deletion of CB1R in neonatal mice exposed to ethanol because the protein kinase A/cAMP/ERK pathway has been shown to phosphorylate CREB on Ser133; ethanol-induced inhibition of this pathway may be responsible for the observed deficits in CREB phosphorylation. It should be noted that CREB has been shown to mediate adaptive responses of neurons to several stimuli to regulate neuronal survival in the developing brain (Bonni et al., 1999). Interestingly, CB1R neonatal KO mice exhibit high levels of CREB phosphorylation similar to adult CB1RKO mice compared to their WT littermates (Basavarajappa et al., 2014; Basavarajappa and Subbanna, 2014). Activated CREB has been shown to regulate the expression of many genes involved in numerous cellular functions, including neuronal survival, synaptic plasticity, and learning and memory (Nonaka, 2009; Benito and Barco, 2010; Sakamoto et al., 2011). Therefore, ethanol-caused dysregulation of this pathway during postnatal development to adulthood may significantly contribute to long-term neurobehavioral deficits commonly associated with FASD (Izumi et al., 2005; Wilson et al., 2011; Sadrian et al., 2012; Subbanna et al., 2013a).

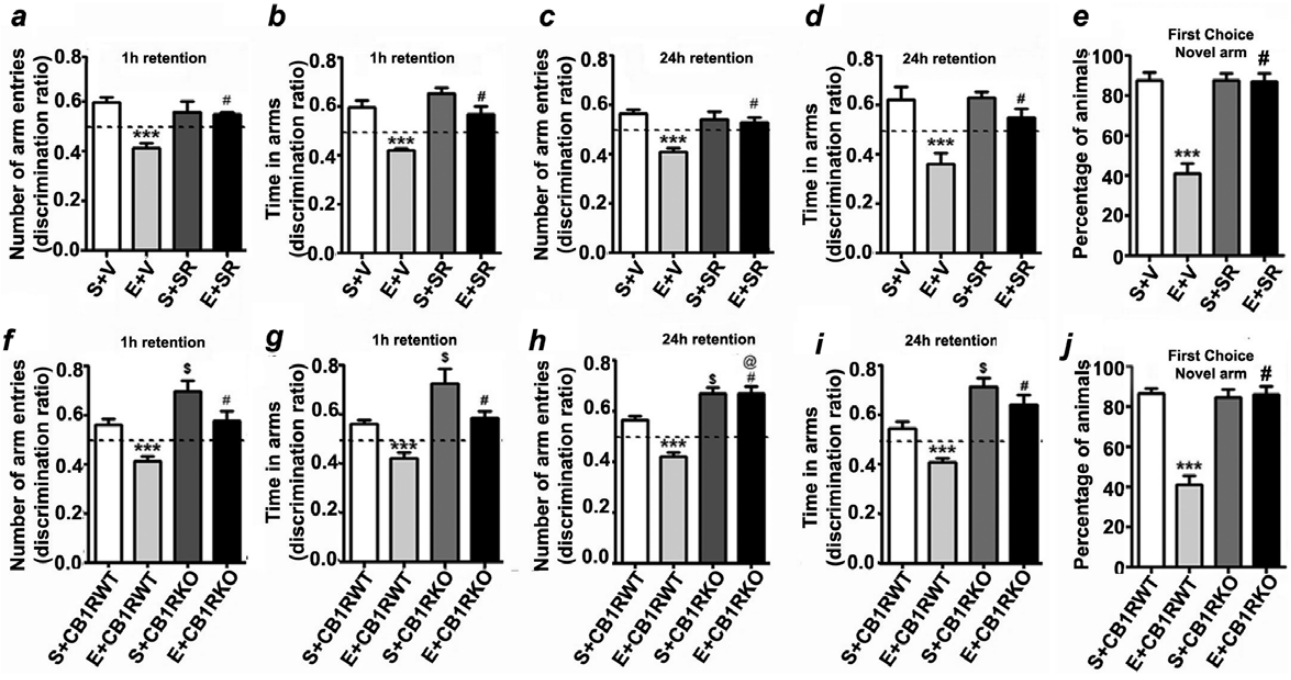

Figure 8. P7 ethanol treatment impairs and SR pretreatment rescues impaired spatial memory performance as measured by the Y maze. Discrimination ratio (preference for the novel arm over the familiar or other arm: Novel/Novel + Other) for arm entries (a and c) and dwell time (b and d) of S+V- and E+V-treated mice with or without SR in the Y maze at 1 h and 24 h after the first encounter with the partially-opened maze. The dashed line indicates chance performance (0.5). (e) The percentage of animals selecting the novel arm as the first choice is shown for S+V- and E+V-treated mice with or without SR at 1 h and 24 h after the first encounter with the partially-opened maze. Each point is the mean ± SEM (n = 8 mice/group). Two-way ANOVA with Bonferroni's post hoc test: ***$p < 0.001$ vs. S + V; #$p < 0.05$ vs. E + V. Discrimination ratio (preference for the novel arm over the familiar or other arm: Novel/Novel + Other) for arm entries (f and h) and dwell time (g and i) of S+CB1RWT, E+CB1RWT, S+CB1RKO, and E+CB1RKO mice in the Y maze at 1 h and 24 h after the first encounter with the partially-opened maze. The dashed line indicates chance performance (0.5). (j) The percentage of animals selecting the novel arm as the first choice is shown for S+CB1RWT, E+CB1RWT, S+CB1RKO, and E+CB1RKO mice at 1 h and 24 h after the first encounter with the partially-opened maze. Each point is the mean ± SEM (n = 8 mice/group). Two-way ANOVA with Bonferroni's post hoc test: ***$p < 0.001$ vs. S + V; #$p \leq 0.01$ vs. E ± V; \$$p \leq 0.01$ vs. S ± CB1RWT; @$p \leq 0.01$ vs. S ± CB1RWT.

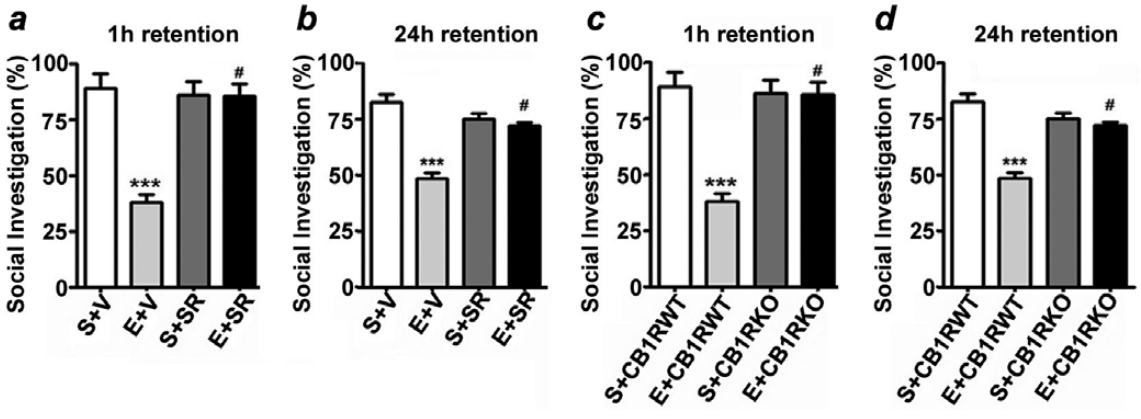

Figure 9. SR pretreatment rescues and CB1RKO provides protection against P7 ethanol-induced social recognition memory loss in adult mice. The percentage of social investigation is shown for S+V-, E+V-, S+SR-, and E+ SR-treated mice at 1 h (a) and 24 h (b) after the first encounter with the same juvenile mouse. ***$p < 0.001$ vs. S + V; # $p < 0.05$ vs. E + V. The percentage of social investigation is shown for S+CB1RWT, E+CB1RWT, S+CB1RKO, and E+CB1RKO mice at 1 h (c) and 24 h (d) after the first encounter with the same juvenile mouse. Each point is the mean ± SEM (n = 8 mice/group). Two-way ANOVA with Bonferroni's post hoc test: ***$p < 0.001$ vs. CB1RWT + S; #$p < 0.05$ vs. CB1RWT + E.

While the precise signaling cascades involved in Arc transcription are not well defined, one study has shown that PKA/MAPK cascades regulate Arc expression (Waltereit et al., 2001) and that the MAPK/CREB pathway is also essential for Arc expression (Ying et al., 2002; Nonaka et al., 2014). Given the impaired ERK1/2 and pCREB in postnatal ethanol-exposed neonatal mice, Arc protein levels were also significantly reduced in ethanol-exposed neonatal as well as adult mice and blockade or genetic deletion of CB1R prior to P7 ethanol treatment–restored Arc protein levels in neonatal and adult mice. Interestingly, both neonatal and adult CB1R KO mice expressed high levels of Arc compared to their WT littermates. It is therefore possible that the neuroprotective role of the CB1R antagonist in neonatal mice may be due to the activation of Arc expression through CB1R-mediated CREB pathway. Arc expression is very tightly controlled by

neuronal activity downstream of multiple signaling pathways (Bramham et al., 2008; Shepherd and Bear, 2011). Therefore, several molecules responsible for mediating neural activity, such as NMDA and the P/Q-type voltage-dependent Ca^{2+} channel, also regulate Arc expression (Kakizawa et al., 2000; Hashimoto et al., 2011). To our knowledge, this is the first study to suggest that the regulation of Arc expression through CB1R activity in neonatal and adult mice. Arc transcription is also regulated by voltage-sensitive calcium channels (Adams et al., 2009), which are negatively regulated by CB1R (Basavarajappa and Arancio, 2008). Our findings suggest that the ERK1/2-pCREB-Arc pathway is involved in neuronal survival downstream of the CB1Rs in the developing brain and is compromised by ethanol treatment. It is possible that ethanol-induced suppression of the ERK1/2-pCREB-Arc signaling pathway might disrupt the fine-tuning of neuronal circuits, leading to persistent synaptic and memory dysfunction (Subbanna et al., 2013a). Consistent with this notion, CB1RKO mice do not exhibit P7 ethanol-induced neurodegeneration during the neonatal period (Hansen et al., 2008; Subbanna et al., 2013a) or deficits in long-term potentiation and ORT during adulthood (Subbanna et al., 2013a).

The current findings also revealed significant deficits in learning and memory in adult mice exposed to ethanol at P7 compared to controls, as tested in several domains (spontaneous alternation, spatial, and social recognition). These findings are in general agreement with previous reports showing that an acute dose of ethanol at P7 impairs synaptic plasticity in the HP (Izumi et al., 2005; Sadrian et al., 2012; Subbanna et al., 2013a) and olfacto-HP (Wilson et al., 2011; Sadrian et al., 2012) circuits as well as ORT (Subbanna et al., 2013a) in adult mice. Most importantly, we have shown that the blockade of CB1Rs before ethanol exposure is sufficient enough to rescue ethanol-induced neuronal deficits in every paradigm we have examined. Several other rodent models of FASD also show impaired learning and memory in adult rodents exposed to acute or chronic ethanol at pre- or postnatal stages of development (Girard et al., 2000; Savage et al., 2002; Christie et al., 2005; Iqbal et al., 2006; Thomas et al., 2008). There is also growing evidence that heavy prenatal alcohol exposure leads to widespread cognitive deficits in children across several domains, including memory and social and adaptive functioning (Norman et al., 2013).

The findings from SRM studies are in general agreement with other studies in which acute exposure to ethanol on P12 caused pronounced and permanent deficits in social behavior throughout ontogeny (Mooney and Varlinskaya, 2011). Similar SRM deficits were also found in another animal model of FASD (Shirasaka et al., 2012), as well as in the CD38 KO model of autism (Jin et al., 2007). Most importantly, SR pretreatment and genetic deletion of CB1R provided protection against ethanol-induced deficits in SRM in adult mice. It has been suggested that retaining normal SRMs throughout ontogeny would help to establish relationships within a group or between partners, besides developing the ability to recognize families (Cushing and Kramer, 2005). Evidence suggests that the two brain regions, the olfactory system (Sanchez-Andrade and Kendrick, 2009) and the limbic system (Brothers et al., 1990; Baron-Cohen et al., 1994) regulate social behavior. In ethanol-treated P7 mice, improper processing of socially relevant olfactory stimuli might produce the observed deficit in SRM in adult mice. Early ethanol exposure damages olfactory neuroanatomy and physiology in both humans and rodents (Wilson et al., 2011). The olfactory system provides a major input to the HP formation (Wilson and Sullivan, 2011), and this structure is involved in integrating the complex stimuli necessary for the recognition process (Alvarez et al., 2002; Ross and Eichenbaum, 2006), therefore the SRM might be regulated also by HP structure (Kogan et al., 2000). Consistent with this notion, our previous findings suggest that ethanol treatment of P7 mice significantly impairs olfacto-HP system function in adult mice (Wilson et al., 2011; Sadrian et al., 2012).

In conclusion, CB1R gene transcription is regulated by specific epigenetic modification, associated with active transcription leading to enhanced CB1R function. Our current findings directly pinpoint the participation of CB1R signaling during early brain development (Figure 10) leading to long-lasting pCREB-Arc impairments and neurobehavioral abnormalities. Currently, effective treatment for individuals suffering from FASD is not available. The CB1R-pCREB-Arc mediated molecular mechanisms in the effect of postnatal ethanol on abnormalities in neuronal survival and its long-lasting influence on synaptic plasticity, learning, and memory, including SRM, may eventually lead to the development of drugs to improve specific aspects of the symptomatology of ethanol-induced neurobehavioral teratogenicity.

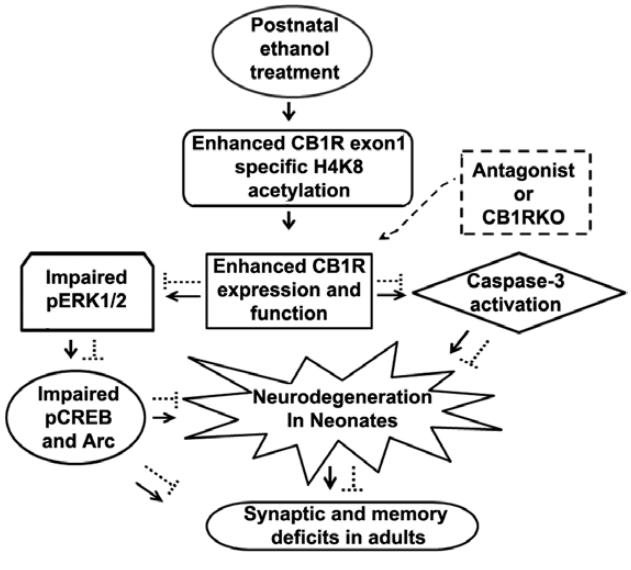

Figure 10. A schematic diagram of the proposed mechanism of action by which epigenetically enhanced CB1R function in neonatal mice regulates ethanol-induced neurodegeneration, leading to neurobehavioral abnormalities in adult mice. P7 ethanol enhances specific H4K8ace of CB1Rs promoter to enhance CB1R expression and function, which causes caspase-3 activation as well as pERK1/2 (Subbanna et al., 2013a), pCREB and Arc deficits and leads to neonatal neurodegeneration (→). These mechanisms during postnatal development may disrupt the refinement of neuronal circuits (Wilson et al., 2011; Sadrian et al., 2012) and lead to long-lasting deficits in synaptic plasticity (Subbanna et al., 2013a), learning, and memory, including social recognition memory in adult animals. The inhibition of CB1Rs (-----) rescue pERK1/2, pCREB, and Arc deficits as well as neonatal neurodegeneration (caspase-3 cleavage), which results in normal neurobehavioral function in adult mice. The genetic ablation of CB1Rs (-----) provides protection against ethanol-induced pCREB, Arc deficits, neonatal neurodegeneration, synaptic (Subbanna et al., 2013a), learning, and memory, including social recognition memory deficits in adult mice. Hence, the putative CB1R/pERK1/2/pCREB/Arc signaling mechanism may have a potential regulatory role in neuronal function during brain development and may be a valuable therapeutic target for FASD.

Acknowledgments

This work in part was supported by NIH/NIAAA grant AA019443 (Dr Basavarajappa). We thank Neha Balapal for editing the final version of the paper.

Statement of Interest

The authors declare no conflicts of interest.

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