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Acute ethanol inhibition of adult hippocampal neurogenesis involves CB1 cannabinoid receptor signaling

Dal Khatri, MS[1,*], **Genevieve Laroche, PhD**[1,*], **Marion L. Grant, BS**[2], **Victoria M. Jones, BS**[3], **Ryan P. Vetreno, PhD**[4], **Fulton T. Crews, PhD**[4,5], and **Somnath Mukhopadhyay, PhD**[1,3,5,#]

[1]Neuroscience Research Program, Biomedical Biotechnology Research Institute, North Carolina Central University, Durham, NC 27707

[2]Department of Biology, North Carolina Central University, Durham, NC 27707

[3]Department of Chemistry & Biochemistry, North Carolina Central University, Durham, NC 27707, Biomedical Biotechnology Research Institute, North Carolina Central University, Durham, NC 27707

[4]Bowles Alcohol Research Center, UNC-Chapel Hill School of Medicine, Chapel Hill NC 27599

[5]Department of Pharmacology, UNC-Chapel Hill School of Medicine, Chapel Hill NC 27599

Abstract

Background—Chronic ethanol exposure has been found to inhibit adult hippocampal neurogenesis in multiple models of alcohol addiction. However, acute ethanol inhibition of adult neurogenesis is not well studied. Although many abused drugs have been found to inhibit adult neurogenesis, few have studied cannabinoids or cannabinoids with ethanol, although human use of both together is becoming more common. We used an acute binge alcohol drinking model in combination with select cannabinoid receptor agonists and antagonists to investigate the actions of each alone and together on hippocampal neurogenesis.

Methods—Adult male Wistar rats were treated with an acute binge dose of ethanol (5 g/kg, i.g.), CB1R or CB2R agonists, as well as selective CB antagonists, alone or combined. Hippocampal doublecortin, Ki67 and activated cleaved caspase-3 (CC3) immunohistochemistry were used to assess neurogenesis, neuroprogenitor proliferation and cell death respectively.

Results—We found that treatment with ethanol or the CB1R agonist, ACEA, and the combination significantly reduced doublecortin positive neurons (DCX+IR) in dentate gyrus and increased CC3. Further, using an inhibitor of endocannabinoid metabolism, e.g. JZL195, we also found reduced DCX+IR neurogenesis. Treatment with 2 different CB1R antagonists (AM251 or SR141716) reversed both CB1R agonist and ethanol inhibition of adult neurogenesis. CB2R agonist HU-308 treatment did not produce any significant change in DCX+IR. Interestingly, neither ethanol nor CB1R agonist produced any alteration in cell proliferation in dentate gyrus as

[#]Corresponding author: Somnath Mukhopadhyay, PhD, Associate Professor, Neuroscience Research Program, Biomedical/ Biotechnology Research Institute, North Carolina Central University, 700 George Street, Durham, NC 27707, USA, Phone: 919-530-7762; Fax: 919-530-7998, smukhopadhyay@nccu.edu.

[*]These two authors equally contributed in this paper

measured by Ki67+ cell population, but cleaved caspase-3 positive cell numbers increased following ethanol or ACEA treatment suggesting an increase in cell death.

Conclusion—Together, these findings suggest that acute CB1R cannabinoid receptor activation and binge ethanol treatment reduce neurogenesis through mechanisms involving CB1R.

Keywords

cannabinoid receptor; ethanol; alcohol; adult neurogenesis; dentate gyrus

Introduction

The extensive use of cannabis is likely to increase exposure of cannabinoids and alcohol during social and other occasions. The endocannabinoid (eCB) system in brain contributes to pain, appetite, and cognition (Dalton and Howlett, 2012) and is known to disrupt hippocampus-dependent behavior (Katona and Freund, 2012). It includes two key receptors, e.g. CB1R and CB2R cannabinoid receptors that respond to plant cannabinoids as well as endogenous cannabinoid (eCB) agonists. eCB signaling can be modified by pharmacological inhibition of synthetic or catabolic breakdown enzymes. For example, the administration of the URB597 (endocannabinoid degrading enzyme, FAAH (fatty acid amide hydrolase) inhibitor) into the prefrontal cortex has been shown to increase alcohol intake in rodents (Basavarajappa et al., 2006, Hansson et al., 2007, Vinod et al., 2008). However, discrepancies in animal models findings confound the effects of the modulators of endocannabinoid signaling on alcohol consumption (Serrano and Parsons, 2011). FAAH inhibition has also been reported to reduce alcohol withdrawal in rats (Cippitelli et al., 2008). Endocannabinoids are known to have major actions on glutamatergic signaling, similar to ethanol and to increase fetal alcohol-induced neurodegeneration (Basavarajappa, 2015). Alternatively, eCB have been postulated to be neuroprotective in ethanol-induced neural progenitor cell culture studies (Katona and Freund, 2012, Galve-Roperh et al., 2013, Bandiera et al., 2014).The eCB and ethanol responses vary across cells in part due to differences in CB1R and CB2R expression, with CB1R the predominant adult brain CB receptor, with CB2R postulated to be expressed in microglia. Previous studies have shown extensive involvement of eCB signaling in brain development and found eCB signals interact with ethanol-induced changes in neurons and glia that contribute to fetal alcohol-induced pathology (Basavarajappa, 2015).These studies prompted an investigation of how ethanol and eCB might interact to impact neural progenitor cells (NPC) and neurogenesis in adult brain.

A substantial literature across multiple species has investigated the formation of new neurons, e.g. neurogenesis, in adult brain, with a focus on hippocampal dentate gyrus. Multiple models of ethanol exposure, particularly, chronic binge models have linked ethanol inhibition of neurogenesis to alcohol induced neurodegeneration (Crews and Nixon, 2009). Further, several studies have well documented that alcohol-induced neurodegeneration resulted in cognitive disorders due to apoptosis of neuronal cells and/or the decrease in adult brain neurogenesis following an acute and chronic alcohol consumption (Nixon and Crews, 2004). More recent studies have associated a persistent loss of both dorsal and ventral hippocampal neurogenesis following adolescent ethanol exposure with adult cognitive

dysfunction (Vetreno and Crews, 2015). Alternatively, in aged mouse brain, activation of CB1R and/or CB2R or the inhibition of the eCB anandamide degrading enzyme FAAH, promotes neural proliferation and neurogenesis that is blocked by the CB1R antagonist SR141716 (SR1) (Aguado et al., 2005, Goncalves et al., 2008).This is consistent with studies suggesting CB1R activation promotes neuronal differentiation, maturation and resistance to brain injury (Parmentier-Batteur et al., 2002, Marsicano et al., 2003, Fowler et al., 2010, Compagnucci et al., 2013). Recently, selective CB2R activation has also been shown to protect deficits in neurogenesis, without the undesired psychoactive effects of neuronal CB1R activation (Stella, 2010, Palazuelos et al., 2012, Rom and Persidsky, 2013, Avraham et al., 2014).Thus the effects and mechanism of the activation of eCB signaling on alcohol consumption and/or the ethanol-mediated effects in brain are not clear.

Most studies of ethanol induced changes in adult neurogenesis have investigated chronic ethanol treatment, however, human use of both cannabis and alcohol are likely to be combined acute exposures. To understand if combined acute CB receptor activation and acute binge ethanol altered adult hippocampal neurogenesis we employ known CB receptor agonists, antagonists and a FAAH inhibitor combined with an acute binge ethanol treatment and determine neurogenesis after 3 hr and 24 hr. We report here that exogenous activation of CB1R and inhibition of eCB catabolism alone or in combination with binge ethanol resulted in robust inhibition of neurogenesis in hippocampal dentate gyrus that coincides with a marked increase in markers of activated caspase3 (a marker of cell death). Further, cotreatment with CB1R antagonists (AM251 or SR141716), but not CB2, block both ethanol or CB1R agonist (alone or in combination)-induced inhibition of adult neurogenesis and increased expression of cell death markers. These findings suggest ethanol and CB1Rs interact to inhibit adult neurogenesis during acute alcohol intoxication.

Materials And Methods

Drugs

ACEA (Arachidonoyl 2′-Chloroethylamide), **JZL195** (4-[(3-Phenoxyphenyl) methyl]-1-piperazinecarboxylic acid 4-nitrophenyl ester), **SR141716** (5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-Methyl-N-1-piperidinyl-1H-pyrazole-3-carboxamide), **AM251**(N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide) and **HU308** 4-[4-(1,1-dimethylheptyl)-2,6-dimethoxyphenyl]-6,6-dimethyl-bicyclo [3.1.1] hept-2-ene-2-methanol, were purchased from Cayman Chemicals (Ann Arbor, Michigan, USA).

Animals and drugs treatment

Adult male Wistar rats (~3 months of age; 280-320 g) were acclimated to the animal facility for seven days at the University of North Carolina at Chapel Hill and randomly assigned to treatment groups (n = 6 – 8 subject per group) prior to the onset of experimentation. All animals were pair housed standard housing cages (GR900; floor area: 904 cm^2; Tecniplast, West Chester, PA) in a temperature- (20°C) and humidity-controlled (30% - 70%) vivarium on a 12 hr/12 hr light/dark cycle (light onset at 0700 hr), and provided *ad libitum* access to food and water. Experimental procedures were approved by the Institutional Animal Care

and Use Committee (IACUC) of the University of North Carolina at Chapel Hill, and conducted in accordance with National Institutes of Health (NIH) regulations for the care and use of animals in research.

For the ethanol experiment, subjects received either a single dose of ethanol (EtOH; 5.0 g/kg, i.g., 25% w/v in H_2O) at a volume of 0.025 mL/g body weight to produce a blood ethanol level of 100-160 mg% as previously described (Crews et.al., 2006) or a comparable volume of Vehicle.

For the cannabinoid experiments, all drugs were purchased from Cayman Chemical (Ann Arbor, MI) and stock solutions prepared as recommended by the manufacturer. SR141716 (SR1; 1.0 mg/kg, i.p.) and AM251 (1.0 mg/kg, i.p.) were supplied as a solid and dissolved in minimum volume of ethanol to prepare a stock solution (30 mg/mL and 14 mg/mL, respectively). JZL 195 crystal was dissolved in DMSO to produce a stock solution of 1.25 mg/mL and administered at 3.0 mg/kg, i.p. cannabinoid receptor type 1 (CB1R) agonist ACEA (3.0 mg/kg, i.p.) was supplied in methyl acetate and the organic solvent evaporated under nitrogen and dissolved in ethanol to produce a 20 mg/mL stock solution. The cannabinoid receptor type 2 (CB2) agonist HU308 was administered at 15 mg/kg, i.p. All drugs were further diluted using a vehicle consisting of 5.0% ethanol, 5.0% emulphor (alkamuls-620; Rhone-Poulenc, Princeton, NJ), and 90% saline (0.9%; ethanol: emulphor: saline 1: 1: 18; (Wiley et al., 2006; Kinsey et al., 2013; Wiebelhaus et al., 2014; Gamage et al 2014). All cannabinoid compounds were administered intraperitoneally (i.p.) at a volume of 10 μL/g body mass. All solutions were warmed to room temperature prior to injection. The control group received a comparable i.p. injection of the same vehicle. The doses of the drugs were chosen based on the literature search for the *in vivo* doses of the compounds used in other studies (Wilson et al., 2006, Wiley et al., 2006, Tsvetanova 2006, Walsh 2015). CB1R antagonist SR1 and AM251 was administered 30 minutes prior to ethanol and/or CB1R agonist ACEA or JZL 195 administration in all experiments. The amount of ethanol present in the vehicle was insignificant after drug dilution. Animals were sacrificed 3 hr or 24 hr after the conclusion of treatment.

Animal perfusion, tissue preparation and immunohistochemistry

Perfusion and brain tissue preparation and immunohistochemistry procedures were performed as described early (Vetreno & Crews, 2015). Briefly, at the end of each experiment, animals were euthanized with sodium pentobarbital (100 mg/kg, i.p.) and transcardially perfused with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 4.0% paraformaldehyde in PBS. Brains were excised quickly and post-fixed in 4.0% paraformaldehyde for 24 h at 4 °C followed by 4 days of fixation in 30% sucrose solution. Hippocampal tissue sections were sampled between bregma -2.12 mm and -3.80 mm according to the atlas of Paxinos and Watson (1998) yielding tissue for analysis of the dentate gyrus throughout the dorsal hippocampus. Coronal sections (1:12 series) of 40 μm were collected on a vibrating microtome (Leica VT1000S) and stored in cryoprotectant (30% glycol/30% ethylene glycol in PBS) at −20 °C until immunohistochemical labeling. Free-floating sections (every 12^{th} section) were processed for immunohistochemistry following standard procedures for DAB detection. Briefly, tissue was washed in 0.1M PBS,

incubated in 0.3% H_2O_2 in PBS to inhibit endogenous peroxidases, and incubated in a blocking solution (PBS, 0.1% Triton-X and 4% goat serum [MP Biomedicals, Solon, OH]) for 1 h. Tissue was then incubated at 4 °C for 24 h in primary antibody (anti-DCX; 1:1000) or anti-Ki67; 1:500) from Abcam) diluted in blocking solution. Sections were then washed with PBS, incubated in biotinylated goat anti-rabbit IgG for 1 h (Vector Laboratories, Burlingame, CA), and incubated for 1 hr in avidin-biotin complex solution (Vectastain ABC Kit; Vector Laboratories). The chromogen, nickel-enhanced diaminobenzidine (Sigma-Aldrich, St. Louis, MO), was used to visualize immunoreactivity. Tissue was mounted onto slides, dehydrated, and cover slipped. Negative control for non-specific binding was conducted on separate sections employing the abovementioned procedures omitting the primary antibody.

For cleaved caspase staining, standard immunofluorescence procedure was followed. The free-floating sections (every12th section) were washed in 0.1 M PBS for 15 minutes thrice. The tissues were then transferred into permeabilization buffer (0.2% TritonX-100 in PBS) and incubated for 2 hr. at room temperature, blocked in a blocking solution (10% goat serum,0.05% Triton X-100 and PBS) for 2 hr. at 37°C. Sections were then incubated in cleaved caspase primary Ab (Cell signaling # 9661; for 48 hr at 4°C followed by washing in wash buffer (PBS,0.01%Triton X-100,1% goat serum). This cleaved caspase-3 (Asp175) antibody has been validated by the manufacturer using Western blot as well as immunohistochemistry. It detects a large fragment (17/19 kDa) of activated caspase-3 resulting from cleavage adjacent to Asp175 of the inactive globulin CC3. The antibody does not recognize inactive full length caspase-3 or other cleaved caspases) in tissue sections (Gown et al.,2002, Kaiser et al., 2008, Olney et al., 2002, Yang et al., 2017). The sections were then incubated in Alexa Fluor 488-Goat Anti-Rabbit IgG (Jackson Immunoresearch, West Grove, PA) for 48 hr at 4°C followed by washing in PBS thrice for 15 minutes each. After the washes, the sections were mounted on glass slides using Vectashield mounting media containing DAPI (Vector Laboratories, Inc. Burlingame, CA). The slides were coversliped, edges sealed with nail polish and let dry. The slides were then stored at 4°C and the images taken using a fluorescence microscope.

Image acquisition and analysis

For this entire study, Bioquant Nova Advanced Image Analysis (R&M Biometric, Nashville, TN) was used for image capture and analysis. Images were captured with an Olympus BX50 microscope and Sony DXC-390 video camera. Quantification of the sections were carried out following the method described early (Vetreno and Crews, 2015) using an Olympus microscope equipped with an Olympus UPlan objective (10×/0.30). Assessment of DCX was performed throughout the dorsal and ventral hippocampal dentate gyrus according to the atlas of Paxinos and Watson, 1998. The total number of Ki-67 and cleaved caspase-3 immunoreactive cells were quantified throughout the dorsal hippocampus, and data are expressed as cells per mm^2.

Statistical analysis

Statistical analyses were performed using Graph Pad Prism software (version 5.0) and One-way ANOVA (Analysis of variance) and post-hoc analyses were performed using Bonferroni test. Data are expressed as mean ± SEM, and significance was defined as $p \leq 0.05$.

Results

Neurogenesis is reduced following acute ethanol and cannabinoid treatment

To determine how ethanol and cannabinoid effect adult neurogenesis, we assessed doublecortin (DCX, a protein expressed uniquely in developing, but not mature neurons) +IR cells in dentate gyrus. Acute ethanol exposure decreased DCX+IR by 48% (**p<0.01) (Figure 1A). We then tested ACEA, a selective agonist to the CB1R (Hillard et al., 1999) and found a slightly greater significant decrease in DCX+IR (60%,***p<0.001). Surprisingly, combined (ACEA+EtOH) treatment decreased DCX+IR by 63% (***p<0.001) although additive effects were not observed. These finding indicate both acute ethanol and CB1R agonists can inhibit adult hippocampal neurogenesis.

To further investigate our surprising discovery of CB1R inhibition of adult neurogenesis, rats were treated with the CB1R selective antagonist SR141716 prior to treatment with ACEA or the vehicle. As shown in figure 2, similar to the experiment in figure 1, CB1R agonist treatment reduced DCX+IR by about 59% (***p<0.001), whereas CB1R antagonist SR1 cotreatment blocked the ACEA-induced reduction in DCX+IR, while SR1 had no effect alone. Next, we used JZL195, an inhibitor of FAAH (fatty acid amide hydrolase) and MAGL (monoacylglycerol lipase), the two principal hydrolytic enzymes of the endogenous endocannabinoids AEA (anandamide) and 2-AG (2 arachidonoylglycerol, respectively. Rats treated with JZL195 alone had a 43% decrease in DCX+IR (***p<0.001) consistent with increases in endogenous eCB leading to reductions in neurogenesis. The combination of JZL195 with SR1 reversed JZL195-induced decrease in DCX+IR. Interestingly, rats sacrificed 24 hr. after ethanol or ACEA treatment did not show changes in DCX+IR (Fig 3) and this is consistent with transient actions possibly coincident with peak drug levels. The CB2R agonist HU-308 did not affect DCX+IR at either 3 or 24 hr. (Fig. 3). These results indicate CB1R agonist-induced transient (3 hr. after treatment) decrease in DCX+IR are mediated by the CB1R and thereby can be blocked by CB1R antagonist, SR1.

CB1R antagonist reversed ethanol-induced inhibition of hippocampal adult neurogenesis

To determine the impact of CB1R antagonists on acute ethanol inhibition of neurogenesis, we investigated two different CB1R antagonists, AM251 and SR1. Surprisingly, both AM251 (Fig.4 A and B) and SR1 (Fig.4 C and D) treatment prior to ethanol, prevented the ethanol-induced decrease in DCX+IR. Similar to SR1 alone in Fig.2B, AM251 did not produce any effect on DCX+IR alone (Fig.4B). These findings suggest CB1R antagonists can protect against acute binge ethanol induced decreases in DCX+IR neurogenesis.

Increased cell death, but not NPC proliferation by acute ethanol and/or eCB receptor drugs

Our findings that ACEA, JZL195 and ethanol reduce DCX+IR suggests neurogenesis is reduced since DCX identifies NPC committed to a neuronal differentiation. To better

understand the mechanisms, we assessed hippocampal DG NPC proliferation by using Ki67+IR and a marker of cell death, activated cleaved caspase-3+IR (CC3+IR) where NPC are located in hippocampal dentate gyrus. Ki-67 is an endogenous cell cycle protein expressed in actively dividing cells from G1-phase through M-phase and is commonly use to study cell proliferation (Scholzen and Gerdes, 2000). Previous studies have found acute ethanol decreases the formation of BrdU+ NPC 5 hours after ethanol (5 g/kg, i.g. ethanol; similar to dose used here) exposure decreased proliferation (Nixon and Crews, 2002).To our knowledge, this is the first acute ethanol exposure study or acute ACEA exposure alone or in combination study assessing Ki67+IR in hippocampal dentate gyrus. We did not find any significant change in Ki67+IR following acute ethanol ($p>0.05$), acute ACEA ($p>0.05$) or HU-308 ($p>0.05$) at 3 hr. or 24 hr. (Fig. 5). The lack of change in Ki67+IR after acute ethanol treatment contrasts with long lasting decreases in NPC proliferation following adolescent intermittent chronic ethanol exposure (Liu and Crews, 2017).These finding suggest NPC proliferation is not the mechanism of decreased DCX+IR. We next assessed a marker of cell death pathways, activated cleaved caspase-3. Caspase-3, known as the executioner caspase is formed as an inactive zymogen procaspase that does not have protease activity. Activation of cleaved caspase-3 (CC3) can occur through by both extrinsic (death ligand) and intrinsic (mitochondrial depolarization) cell death pathways. Controls show a basal level of cell death in DG since many progenitors do not mature to neurons under control conditions. Interestingly, we found CB1R agonist ACEA significantly increased the number of CC3+IR (cells/mm^2) by 72% (**$p<0.01$) (Fig.6). Ethanol also increased CC3+IR cells by 68% (**$p<0.01$). Similar to our findings on DCX+IR, the CB1R antagonist SR1 alone did not change CC3+IR cells, but blocked both the ACEA and ethanol-induced increase in CC3+IR cells. These findings suggest both acute ethanol and ACEA increase the cell death marker, CC3+IR, through a mechanism sensitive to SR1 and thus mediated by the CB1R.

Discussion

Although extensive studies have investigated eCB involvement in brain development and neuroprogenitors *in vitro* and *in vivo* (Prenderville et.al., 2015), to our knowledge this is the first study to directly investigate the impact of CB1R agonist ACEA and endocannabinoid enhancing JZL195 on expression of the hippocampal neurogenesis marker DCX. We found that acute ethanol treatment as well as acute ACEA and JZL195 treatment reduce DCX+IR consistent with the reduced adult hippocampal neurogenesis. Previous studies have found an acute ethanol binge exposure reduces BrdU labeling of adult NPC in hippocampus (Nixon and Crews, 2002) and reduces DCX+IR in adolescent rat brain after acute ethanol treatment (Crews et.al., 2006). These findings are consistent with chronic ethanol exposure-induced inhibition of neurogenesis (Crews and Nixon, 2008). We found ACEA, the direct CB1R agonist and JZL195, an inhibitor of endogenous eCB metabolism known to increase endogenous levels of eCB agonists, decrease DCX+IR transiently at 3 hr. after treatment, but not 24 hr. after treatment. Further, the reduction in DCX+IR by both ACEA and JZL195 was blocked by the CB1R antagonist SR1 consistent with the CB1R mediating the reduction in DCX+IR. We did not find an effect of the CB2R agonist, HU-308, on DCX+IR at either 3 or 24 hr. consistent with a selective CB1R acute response. Similarly, acute ethanol inhibition of

DCX+IR was found after 3 hr. of exposure, but not 24 hr. after ethanol treatment, consistent with a transient loss of neurogenesis during intoxication. Surprisingly, we found CB1R antagonists, e.g. SR1 and AM251, blocked acute ethanol inhibition of DCX+IR, without any effect of the antagonists alone. Antagonists to CB1R blocked JZL195-mediated decreases in DCX+IR consistent with eCB reducing neurogenesis through CB1Rs. Similarly, reversal of acute ethanol inhibition of DCX+IR by CB1R antagonists is consistent with ethanol increasing endogenous eCB that activate CB1Rs resulting decrease in DCX+IR (Fig. 7). Our studies find robust acute inhibition of DCX+IR by both ethanol and ACEA. Since both are blocked by CB1R antagonists the simplest interpretation of this data is that acute ethanol and ACEA share a common mechanism requiring CB1Rs. DCX+IR NPC are formed from dividing NPC and many do not survive to mature DG neurons so we determined NPC proliferation with Ki67 and cell death using CC3. We found CC3, but not Ki67, was increased by acute ethanol and ACEA, and both were blocked by SR1, consistent with CB1Rs increasing activation of caspase-3, a marker of cell death, that reduces DCX+IR NPC. Hippocampal neurogenesis has been linked to both cognition and mood, and hippocampal NPC mature to granule cell neurons over about a month. The acute reductions in DCX+IR during intoxication with acute alcohol and/or cannabinoid CB1R agonists suggest these agents alone and in combination could disrupt cognition and mood acutely and perhaps later due to a lack of new neurons from maturing NPC. These studies suggest that acute ethanol and cannabinoids alone and together acutely reduce adult hippocampal neurogenesis involving CB1Rs (Fig. 7).

Many studies have found expression of endogenous cannabinoids as well as CB1R and CB2R in both *in vivo* and *in vitro* models of NPC neurogenesis, however, responses vary across models and primarily involve changes in NPC proliferation (Prenderville et al., 2015) which differs from our finding of no change in Ki67+IR, a marker identifying dividing NPC in adult DG. Previous studies in Sprague-Dawley rats using BrdU labeling find a 40% decrease in newly formed BrdU+IR progenitors 5 hours after acute ethanol (5g/kg, i.g.) (Nixon and Crews, 2002) but in our study in Wistar rats, we found no change in Ki67 3 hr. after ETOH treatment. These are 2 different methods for assessment of progenitor proliferation, there are many more BrdU+IR cells/DG which is incorporated in S phase, than the Ki67+IR cells, which label all actively dividing cells (Peng et al., 2017).Thus, ethanol may impact progenitor proliferation that we did not detect with Ki67+IR.Our findings that ethanol and CB1R activation reduced DCX+IR and increased CC3+IR are consistent with previous studies in post-natal day 7 mice (Subbanna et al., 2013). Acute ethanol treatment of P7 mice led to a rapid induction of the synthetic enzymes producing anandamide and CB1Rs as well as a marked increase in hippocampal CC3, the cell death marker, that was blocked by SR1 and not observed in the transgenic mouse lacking CB1R. These studies suggested that in neonates with immature neuron, ethanol and CB1R activation of caspase-3 and inhibition of the kinase ERK contribute to long lasting brain dysfunction (Subbanna, et.al. 2013). Interestingly, transgenic mice lacking CB1Rs show a slight decrease in basal neurogenesis (Wolf et al., 2010, Dubreucq et al., 2010).Our study in rats, did not find an effect of SR1 the CB1R antagonist, treatment consistent with previous studies, finding no effect on hippocampal DG neurogenesis (Jin et al., 2004) and consistent with little or no basal eCB action on neurogenesis. We were surprised that SR1 and another CB1R antagonist blocked

ethanol inhibition of neurogenesis, suggesting release of eCB signals activating CB1Rs that in turn increase cell death in DCX+ progenitors. Other studies have found chronic THC reduces neurogenesis (Wolf et al., 2010) consistent with our acute CB1R agonist reduction in DCX+IR. Our finding that JZL195 which increases endogenous eCB is blocked by SR1 is consistent with ethanol increasing eCB that act on CB1Rs to reduce neurogenesis. The mechanisms of acute ethanol and CB1R agonist inhibition of neurogenesis and increased CC3 need additional studies to fully understand how CC3 is activated in NPC and other cells.

Previous studies have linked ethanol-mediated inhibition of neurogenesis to activation of NFkB and increases in neuroimmune gene expression (Zou and Crews, 2012 ; Koo et al., 2010). Our findings of decreased DCX+IR, a lack of Ki67+IR change and increased CC3+IR suggest neurogenesis is decreased primarily through a decrease in survival of newly generated neurons. However, there are multiple potential mechanisms by which acute ethanol exposure and eCB might impact DCX+IR and CC3+IR. This is the first report of specific CB1R agonists on DCX+IR and CC3+IR. Additional studies are needed to determine if this interaction involves changes in proinflammatory cytokines and/or other mechanisms. Further, our increase in CC3+IR may reflect increased cell death in mature DG neurons or other cells since some CC3+IR cells are outside the DG. Our findings on CB1R agonists and antagonists do not directly translate to marijuana, since it contains a complex mixture of CB agonist and cannabidiol, which is not a CB agonist and protects against ethanol induced neurodegeneration and loss of neurogenesis (Liput et al., 2013). Our studies presented here support the hypothesis that ethanol inhibition of neurogenesis is linked to activation of CB1Rs through eCB release. However, the CB1R and acute alcohol induced reduction in neurogenesis is transient, suggesting multiple cycles may be necessary for any actions related to development of AUD.

Acknowledgments

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Abbreviations

ACEA	Arachidonyl-2′-chloroethylamide
GPCRs	G-protein-coupled receptors
CB1R	cannabinoid 1 receptor
CB2R	cannabinoid 2 receptor
CB	Cannabinoid
DG	dentate gyrus
NPC	neural progenitor cell
DCX	doublecortin
AM251	(*N*-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide
SR141716 (SR1)	5-(4-Chlorophenyl)-1-(2,4-dichloro-phenyl)-4-methyl-N-(piperidin-1-yl)-1H-pyrazole-3-carboxamide
JZL 195	4-[(3-Phenoxyphenyl)methyl]-1-piperazinecarboxylic acid 4-nitrophenyl ester

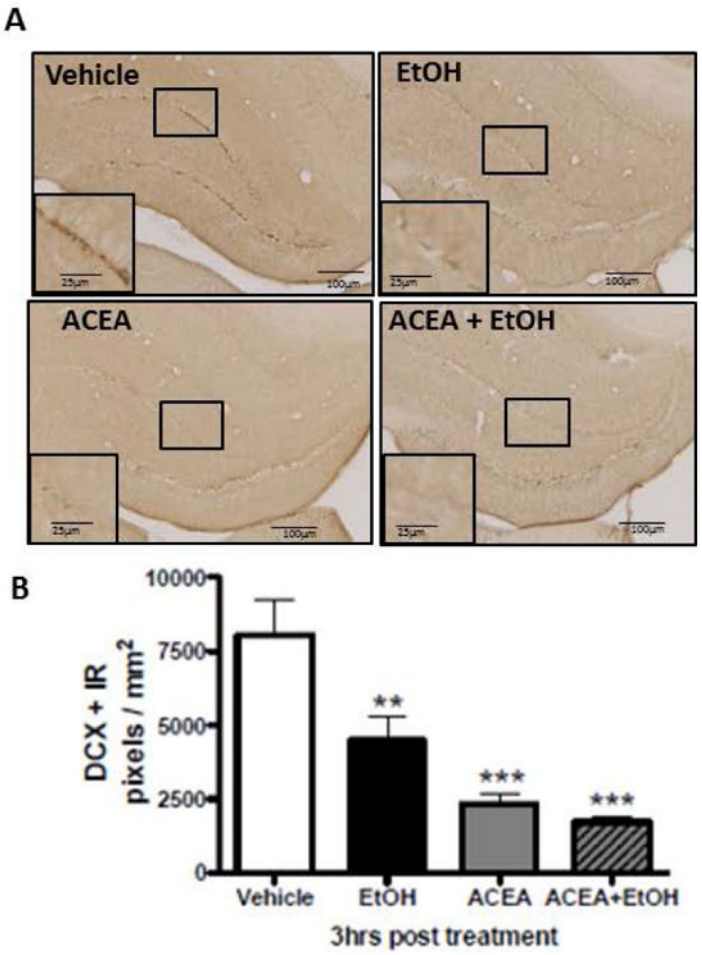

Figure 1. Reduced adult neurogenesis in the dentate gyrus of adult rat hippocampus following acute treatment with CB1R agonist (ACEA) and/or ethanol

A, Representative images (4×; 20× for inlet) of the dentate gyrus with doublecortin-immunopositive neurons (DCX+IR) following acute treatment (3 hr) with CB1R agonist (ACEA, 3 mg/kg, ip) or ethanol (5 g/kg, ig) or combination of both drugs. **B**, Quantification of DCX + IR (pixels/ $mm^{2;}$ n=6). Treatment with EtOH, ACEA alone or in combination (ACEA+EtOH) significantly reduced DCX+IR relative to controls [$F_{(3,87)}$ = 47.72, ***p<0.001].

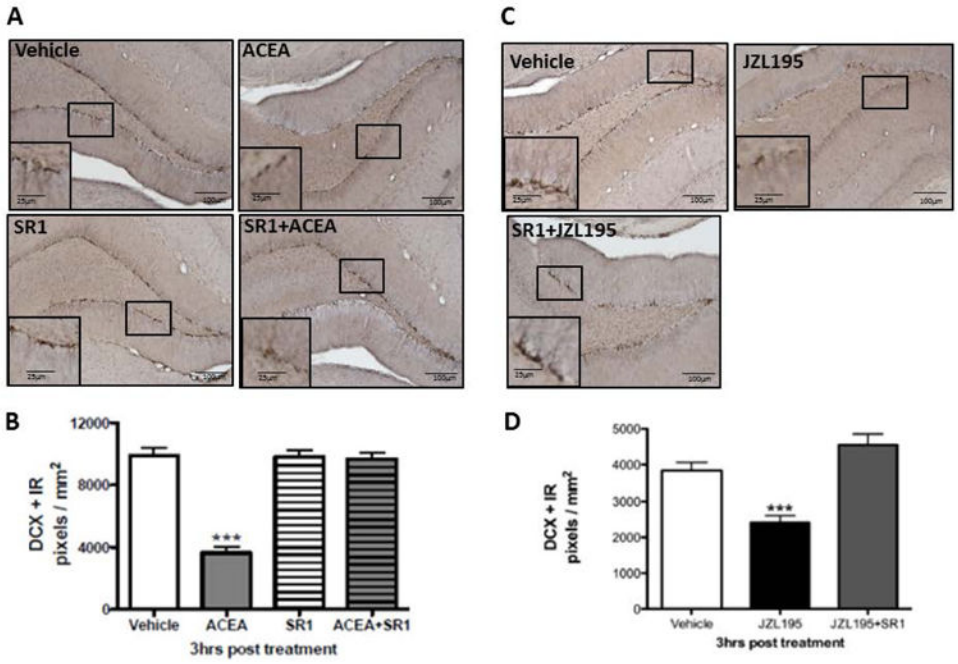

Figure 2. CB1R antagonist pretreatment prevents CB1R agonist ACEA and FAAH-MAGL inhibitor JZL195- mediated inhibition of neurogenesis

A, Images represented DCX +IR neurons following a pre-treatment (20 min before ACEA treatment) with CB1R specific antagonist (SR141716, 3mg/kg, ip) Quantitation of DCX +IR neurons pre-treated (20 min before agonist treatment) with CB1R specific antagonist significantly reversed [$F_{(3,149)}$ = 24.81, ***P<0.001] ACEA-mediated inhibition of neurogenesis. **C, & D** Inhibition of the endocannabinoids inactivation enzymes (FAAH and MAGL) using JZL195 significantly decreased [$F_{(3,104)}$ =18.91, ***p<0.001] adult neurogenesis and CB1R antagonist (SR1) reversed the JZL195-mediated inhibition of neurogenesis.

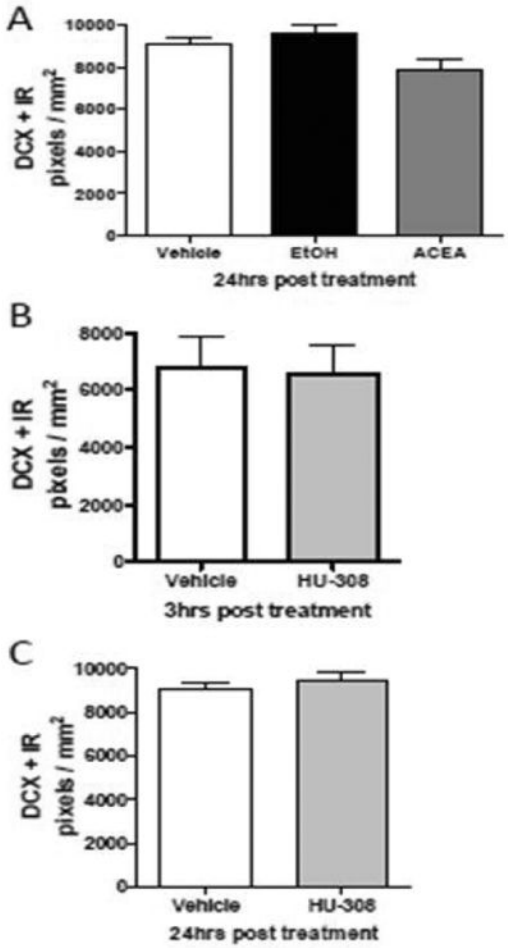

Figure 3. CB2R agonist treatment did not alter adult neurogenesis

A, No significant changes were observed on the number pixels/mm^2 of DCX+IR 24hrr after acute CB1R agonist, ACEA or EtOH treatments. Rats were treated with CB2R selective agonist (HU-308A, 15 mg/kg, ip) and sacrificed after 3 hr (**B**) or 24hrr (**C**). DCX staining quantification did not t show any significant ($p > 0.05$) changes for both post-treatment time point.

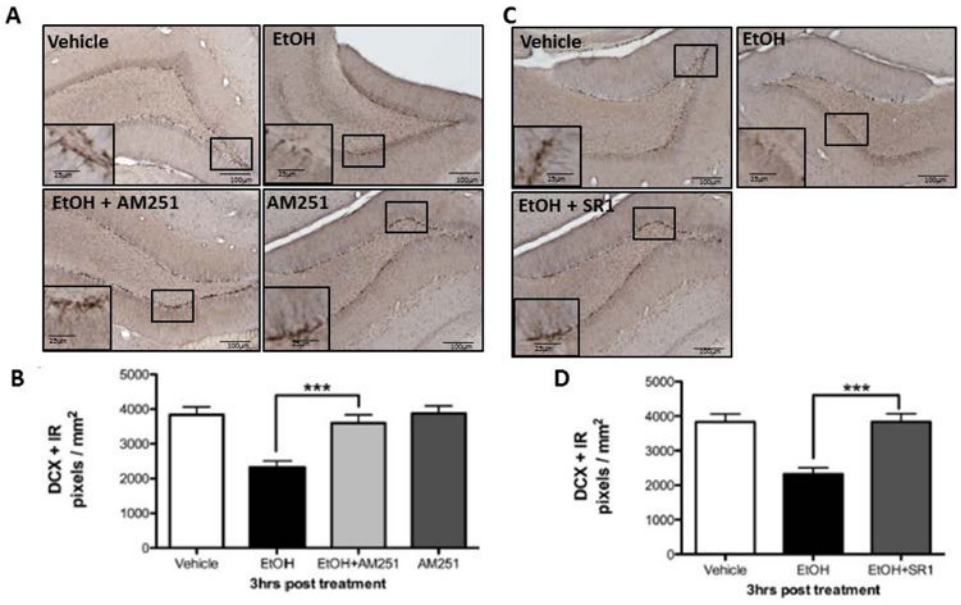

Figure 4. CB1R antagonists treatment reversed binge ethanol-mediated inhibition of adult neurogenesis

A, Images represented DCX +IR neurons following a pre-treatment (20 min before ethanol treatment) with CB1R specific antagonist (AM251, 3mg/kg, ip). **B,** Quantitation of DCX +IR neurons showed pre-treatment with AM251 significantly (***$p<0.001$; n=4) reversed ethanol-mediated inhibition of neurogenesis. **C,** Images represented DCX +IR neurons following a pre-treatment with CB1R specific antagonist (SR141716, 3mg/kg, ip) **D,** Quantitation of DCX +IR neurons showed pre-treatment with SR141716 significantly reversed ethanol-mediated inhibition of neurogenesis. [$F_{(3, 16)} = 22.06$, ***$p<0.001$]

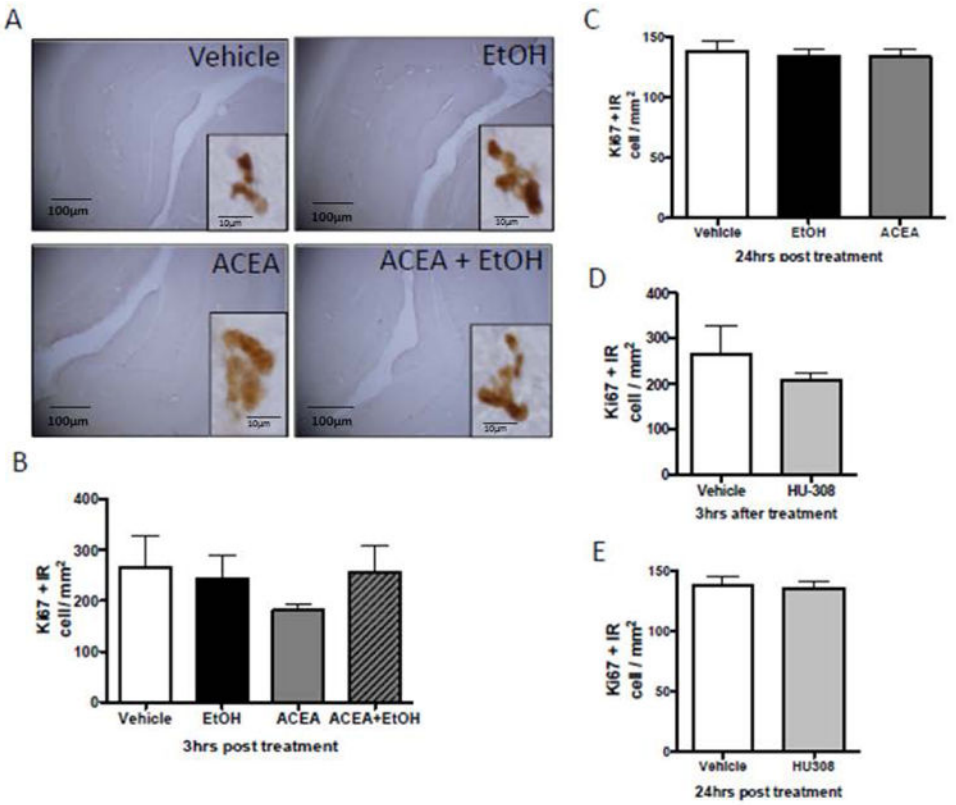

Figure 5. Acute CB1R or CB2R agonists or ethanol treatments did not alter cell proliferation in the dentate gyrus

A, Representative images (4×; 20× for inlet) of dentate gyrus with Ki67+IR (Ki67 positive immunoreactivity) following acute treatment (3 hr) with CB1R agonist (ACEA, 3 mg/kg, ip) or ethanol (5 g/kg, ig) or combination of both drugs. **B**, Quantitation of Ki67+ IR (cells/ $mm^{2;}$ n=6). No significant changes were observed in cell proliferation as measured by Ki67+IR 3 hr **(B)** or 24hrr (**C**) after treatment [$F_{(9,316)} = 1.57$, $p>0.05$]. Rats were also treated with CB2R selective agonist (HU-308, 15 mg/kg, ip) and sacrificed after 3 hr (**D**) or 24hrr (**E**). Ki67 positively stained cells quantification did not show any significant ($p> 0.05$) changes.

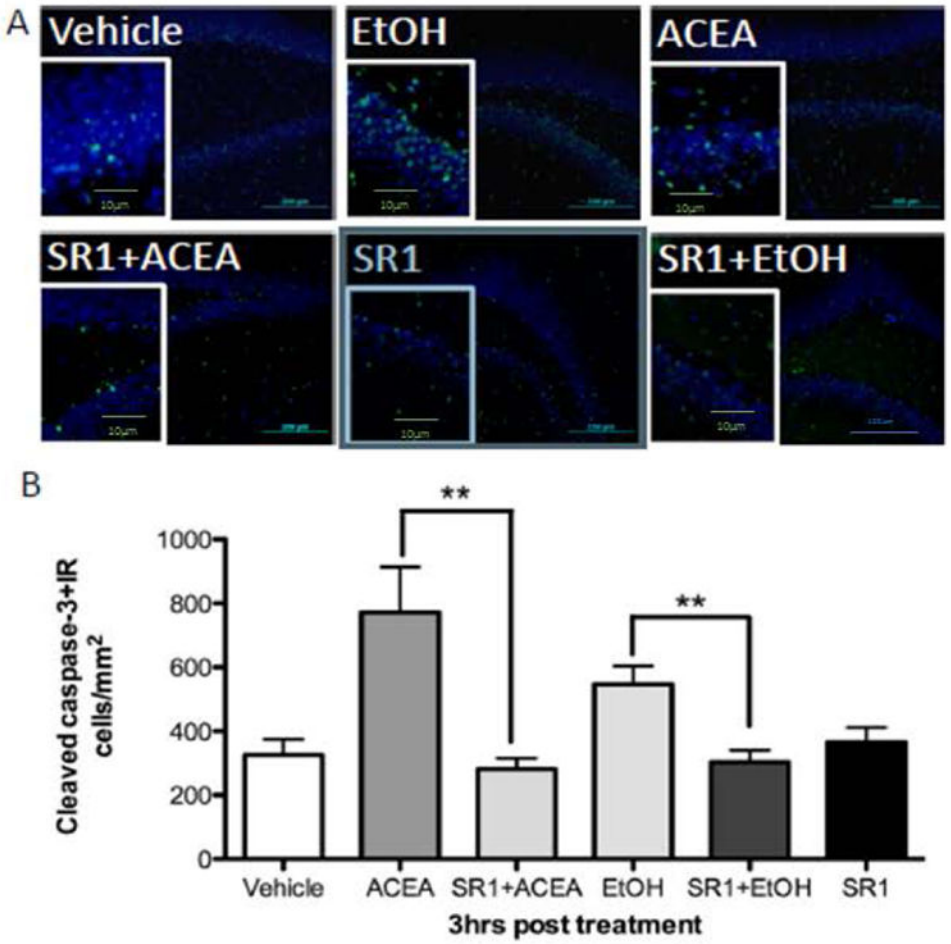

Figure 6. CB1R antagonists blocks binge EtOH and CB1R agonist-mediated apoptosis

A, Representative images (4×; 20× for inlet) of immunofluorescence of the dentate gyrus using the cell death marker cleaved-caspase-3(green) and nuclear DAPI staining (blue) following indicated treatment. **B,** Quantification of the cell number (CC3+IR/mm2) showed a significant increase of cleaved-caspase-3 for animals in the groups treated with ACEA or EtOH relative to controls. [F (5,50=23.46, ***p<0.001]. CB1R antagonist (SR1; 3mg/kg, ip, pre-treatment) reversed ethanol and CB1R agonist ACEA-mediated increase cell death.

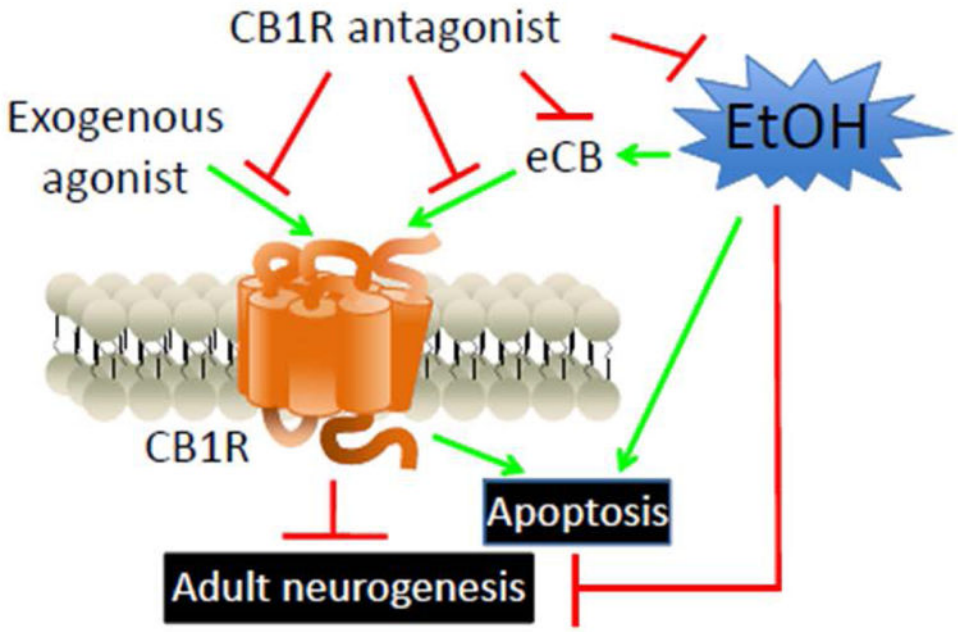

Figure 7.
Schematic of the proposed model of CB1R -mediated regulation ethanol-mediated inhibition of neurogenesis.