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## Maternal alcohol exposure during mid-pregnancy dilates fetal cerebral arteries via endocannabinoid receptors

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### Abstract

Prenatal alcohol exposure often results in fetal alcohol syndrome and fetal alcohol spectrum disorders. Mechanisms of fetal brain damage by alcohol remain unclear. We used baboons (*Papio spp.*) to study alcohol-driven changes in the fetal cerebral artery endocannabinoid system. Pregnant baboons were subjected to binge alcohol exposure via gastric infusion three times during a period equivalent to the second trimester of human pregnancy. A control group was infused with orange-flavored drink that was isocaloric to the alcohol-containing solution. Cesarean sections were performed at a time equivalent to the end of the second trimester of human pregnancy. Fetal cerebral arteries were harvested and subjected to *in vitro* pressurization followed by pharmacological profiling. During each alcohol-infusion episode, maternal blood alcohol concentrations (BAC) reached 80 mg/dL, that is, equivalent to the BAC considered legal intoxication in humans. Circulating anandamide (AEA) and 2-arachidonoylglycerol (2-AG) remained unchanged. Ultrasound studies on pregnant mothers revealed that fetal alcohol exposure decreased peak systolic blood velocity in middle cerebral arteries when compared to pre-alcohol levels. Moreover, ethanol-induced dilation was observed in fetal cerebral arteries pressurized *in vitro*. This dilation was abolished by the mixture of AM251 and AM630, which block cannabinoid

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#### Author contributions

ANB, AMD, and OS designed the research. AT, JSJ, RS, DM, PS, SD, YA, DLT, TC, SB, WL, OS, and ANB acquired data. AT, DM, OS, and ANB analyzed data. OS, AT, JSJ, RS, DM, GM, and ANB wrote the manuscript.

#### Conflict of interest

The authors declare no conflicts of interest.

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receptors 1 and 2, respectively. In the presence of AM251, the cannabinoid receptor agonist AEA evoked a higher, concentration-dependent dilation of cerebral arteries from alcohol-exposed fetuses. The difference in AEA-induced cerebral artery dilation vanished in the presence of AM630. CB1 and CB2 receptor mRNA and protein levels were similar in cerebral arteries from alcohol-exposed and control-exposed fetuses. In summary, alcohol exposure dilates fetal cerebral arteries via endocannabinoid receptors and results in an increased function of CB2.

## Keywords

fetal cerebral artery; baboon pregnancy; maternal alcohol consumption; fetal alcohol exposure; binge drinking during pregnancy

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## Introduction

Alcohol consumption during pregnancy is a serious public health problem and a leading preventable cause of birth defects and developmental disabilities (Basavarajappa, 2015). The adverse effects of alcohol on the developing fetus result in a spectrum of structural abnormalities, behavioral defects, and neurocognitive disabilities termed fetal alcohol spectrum disorders (FASD). The most severe cases are diagnosed as fetal alcohol syndrome (FAS). FASD is estimated to affect 2–5% of children in the U.S. and Western European countries ([www.cdc.gov](http://www.cdc.gov); May & Gossage, 2001; 2011; May et al., 2009). In some regions of the world, however, FAS/FASD affects up to 29% of live births (Olivier, Curfs, & Viljoen, 2016).

It is widely documented that prenatal alcohol (ethanol) exposure causes abnormalities in several organs and systems of the body, such as the heart, kidney, liver, gastrointestinal tract, and the endocrine and immune systems (Caputo, Wood, & Jabbour, 2016; Gauthier, 2015). However, the brain constitutes the most severely affected organ, exhibiting both structural and functional abnormalities in response to prenatal alcohol exposure (Caputo et al., 2016; Mattson, Schoenfeld, & Riley, 2001). Unfortunately, the mechanisms of cerebral damage due to prenatal alcohol exposure are poorly understood and thus, treatment is limited (Murawski, Moore, Thomas, & Riley, 2015).

The developing brain is critically dependent on a sufficient blood supply, which ensures delivery of oxygen and other nutrients. Remarkably, the prevailing neurological symptoms in FAS and FASD, such as memory deficit, low processing speed, lack of cognitive flexibility, and many others are similar to those observed in individuals with progressive neurodegenerative disorders, in which cerebrovascular dysfunction is being increasingly recognized as one of the major pathophysiological mechanisms (Gorelick et al., 2011; Mattson & Riley, 1998; Olson, Feldman, Streissguth, Sampson, & Bookstein, 1998; Zlokovic, 2011).

Endocannabinoid (eCB) signaling is critical for regulating arterial contractility in response to physiological and pathological stimuli (Engeli et al., 2012; López-Miranda, Herradón, & Martín, 2008). The endocannabinoid system (eCBS) consists of eCB-producing enzymes, eCB ligands, eCB receptors (mostly CB1 and CB2 types), and the eCB degradation



acclimate to the new environment at UTHSC. Baboons were singly housed in standard baboon cages, with visual and auditory access to each other. A maximum of four baboons (cages) were housed per room, on a 12-h light/dark cycle (lights on at 6:00 AM) without access to natural light. Baboons were fed twice a day, with each feeding consisting of the High Protein Monkey Diet (~15 biscuits per meal, 21 kcal/biscuit) to sustain the baboons' weight gain as expected throughout the pregnancy. Each feeding was also supplemented by two pieces of fresh fruits or vegetables and two tablespoons of peanut butter. Drinking water was available *ad libitum*. The facilities were maintained in accordance with the USDA and AAALAC standards.

### Alcohol infusion procedure

Dams were randomly assigned to the experimental groups. Dams were subjected to either alcohol or control infusion procedures at 90, 100, and 110 days of gestation as confirmed by an ultrasound exam. Prior to each procedure, the animals were fasted for 12 h. On the day of the experiment, the animal sedation was induced by a single shot of ketamine hydrochloride (Ketaset<sup>®</sup>, 10 mg/kg of body weight). Anesthesia was maintained with isoflurane (1.5–2.0%) throughout the infusion, followed by maternal blood sampling and Doppler evaluation of fetal cerebral artery function. A rectal suppository of indomethacin (25 mg) was used to prevent labor during the alcohol/control infusion. An intravenous (IV) catheter was placed into the saphenous vein for blood collection and another catheter was placed into the cephalic vein for IV sodium chloride infusion. Animal monitoring of vital signs and depth of anesthesia consisted of electrocardiography, pulse oximetry, capnography, non-invasive blood pressure, and temperature measurements. A gastric catheter was introduced into the stomach and the infusion was administered over 10 min. For the experimental group, the infusion contained 1.8 g/kg ethanol (ultrapure, 200 proof; American Bioanalytical) diluted in reverse osmosis-purified drinking water. The control group of animals received an isocaloric solution containing orange-flavored Tang<sup>®</sup> powder (Kraft Foods). In both cases, the total volume of fluid infused was equal to 200 mL. Experimental blood collection occurred at 10 time points (see below) over 180 min, and Doppler evaluation of fetal middle cerebral artery (MCA) function was performed immediately before and at 120 min post-infusion. Before awakening from anesthesia, both groups of animals received a single intramuscular injection of carprofen (Rimadyl, 4.4 mg/kg of body weight), aimed to alleviate symptoms of hangover following alcohol drinking. At 120 days of gestation, Cesarean sections (C-sections) were performed using standard methodology that included maternal anesthesia. During C-section, fetuses were euthanized by exsanguination. In the control group, two fetuses were female and one was male; in the alcohol-exposed group, all four fetuses were male.

### Blood alcohol concentration (BAC) measurements

Blood was collected immediately prior to the gastric infusion of either control or alcohol infusion, and at 10, 30, 60, 75, 90, 105, 120, 160, and 180 min following the infusion episode. Blood samples were collected through an IV catheter inserted into the saphenous vein. Blood alcohol levels were detected using the Nicotinamide Adenine Dinucleotide-Alcohol Dehydrogenase Reagent kit following the manufacturer's instructions (Sigma-Aldrich). Samples and reagent mixture were pipetted into 96-well plates, each sample being

prepared in duplicates. Absorbance was measured at 340 nm using a Synergy microplate reader (BioTek).

### Doppler ultrasonography of fetal MCA

Doppler ultrasonography was performed on anesthetized pregnant baboons immediately prior to and 120 min following gastric infusion of control or alcohol-containing infusion fluid at 90, 100, and 110 days of gestation. At 120 days of gestation, Doppler ultrasonography was performed immediately prior to C-section. Ultrasounds were performed using SonoAce R3 sonographer (Samsung) and GE Voluson E (GE). These sonographers had negligible inter-instrument measurement variability. The MCA was studied just distal to the circle of Willis and the angle of insonation was maintained near 0 degrees. Peak systolic velocity (PSV) and resistive index (RI) were calculated. Each measurement was taken three times, and the average was presented as a single data point.

### Liquid chromatography-tandem mass spectrometry

**Sample preparation**—Fifty  $\mu\text{L}$  of methanol was added to a 50- $\mu\text{L}$  aliquot of plasma sample. The mixture was vortexed for 30 sec, and then centrifuged for 15 min at 4 °C (circa 13,000  $\times g$ ). Seventy-five  $\mu\text{L}$  of supernatant was transferred to another vial containing 50  $\mu\text{L}$  of methanol. The mixture was again vortexed for 30 sec, and centrifuged for 15 min at 4 °C (circa 13,000  $\times g$ ). One hundred  $\mu\text{L}$  of supernatant was carefully transferred into a 96-well plate (max. 400  $\mu\text{L}$  per well). Ten  $\mu\text{L}$  of IS solution (20 ng/mL AEA-d4 and 200 ng/mL 2-AG-d8 in methanol) was added, and an aliquot of 10  $\mu\text{L}$  was injected into the LC-MS/MS system. To build the concentration calibration curve, analyte standards ranging from 0.02 to 50 ng/mL for AEA, and from 0.2 to 500 ng/mL for 2-AG were prepared in methanol solution. Similarly, 100  $\mu\text{L}$  of each standard solution were transferred to a 96-well plate, and 10  $\mu\text{L}$  of IS solution (20 ng/mL AEA-d4 and 200 ng/mL 2-AG-d8 in methanol) was added followed by a 10- $\mu\text{L}$  aliquot injection into the LC-MS/MS system.

**LC-MS/MS Measurements**—LC-MS/MS measurements were carried out using a Triple Quad 5500 tandem mass spectrometer from AB Sciex LLC (Framingham, MA), equipped with a Turbo V™ ion source for electrospray ionization (ESI) with the TurboIonSpray probe, a Shimadzu (Columbia, MD) Nexera XR LC system with CBM20Alite Controller, LC20ADXR pumps, SIL20ACXR AutoSampler, and CTO20AC column oven.

Chromatographic separation of analytes was completed using a Discovery HS C18 column of 50  $\times$  4.6 mm (length  $\times$  internal diameter, and 3- $\mu\text{m}$  particle size (Sigma-Aldrich, St. Louis, MO), maintained at 40 °C. The mobile phases (A: water, and B: methanol, each containing 0.1% formic acid and 2 mM ammonium acetate) were eluted at a flow rate of 0.35 mL/min. The gradient started at 50% of mobile phase B for 0.5 min, then rose to 95% from 0.5 to 1.0 min, and from 1.0 to 6.0 min rose to 100%. Subsequently, the eluent composition was changed to initial condition, 50% of mobile phase B. The total run time was 7 min with an extra 1.0-min equilibration time before each injection. A switching valve directed the mobile phase to the MS system between 3.5–6.5 min. The electrospray ion source was operated in a positive ionization mode. The typical ion source parameters were: capillary 5.5 kV, entrance potential (EP) 10 V, channel electron multiplier (CEM) 2300 V, source temperature 600 °C. Curtain Gas, GS1, and GS2 were set at 20, 60, and 50 psi,

respectively. Quantification was performed in the multiple reaction-monitoring mode (MRM). Analytical data were processed using the software program Analyst (Version 1.6.2).

### **Pressurized fetal cerebral artery diameter monitoring**

Artery diameter monitoring was performed following a general methodology described in our previous work (Bukiya, Vaithianathan, Kuntamallappanavar, Asuncion-Chin, & Dopico, 2011). An arterial segment from the fetal MCA tree was dissected out of the brain and cannulated at each end in a temperature-controlled, custom-made perfusion chamber using a Dynamax RP-1 peristaltic pump (Rainin Instruments, Inc., Oakland, CA). The chamber was continuously perfused at a rate of 3.75 mL/min with physiologic saline solution (PSS) (mM): 119 NaCl, 4.7 KCl, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 1.6 CaCl<sub>2</sub>, 1.2 MgSO<sub>4</sub>, 0.023 EDTA, 11 glucose, and 24 NaHCO<sub>3</sub>. The PSS was equilibrated at pH 7.4 with a 21/5/74% mix of O<sub>2</sub>/CO<sub>2</sub>/N<sub>2</sub> and maintained at 35–37 °C. Arteries were observed with a CCD camera (Sanyo VCB-3512T; Sanyo Electric Co., Moriguchi, Japan) attached to an inverted microscope (Nikon Eclipse TS100; Nikon). The artery external wall diameter was measured using the automatic edge-detection function of IonWizard software (IonOptics, Milton, MA) and was digitized at 1 Hz using a personal computer.

Steady-state changes in intravascular pressure were achieved by elevating an attached reservoir filled with PSS and were monitored using a pressure transducer (Living Systems Instruments, Burlington, VT). Arteries were first incubated at an intravascular pressure of 10 mmHg for 10 min. The intravascular pressure then was increased to 30 mmHg and held steady throughout the experiment to develop and maintain the arterial myogenic tone. Drugs were dissolved to make stock solutions, diluted in PSS to their final concentration, and applied to the artery via chamber perfusion. The effect of a drug application was evaluated at the time it reached a maximal, steady level.

### **Western immunoblotting**

The technique was done as described previously (Zabirnyk, Liu, Khalil, Sharma, & Phang, 2010). Prior to loading, total protein level in each sample was quantified using BCA protein assay kit (Thermo Fisher Scientific, Waltham MA); samples were diluted to yield equal amounts of total protein in each sample. The anti-cannabinoid receptor 1 (ab3559; Abcam, Cambridge, UK) and anti-cannabinoid receptor 2 (ab3560; Abcam, Cambridge, UK) antibodies were used according to the manufacturer recommendations. Specificity of the anti-CB receptor antibodies was validated using non-transfected HEK cells versus adult mouse whole-brain lysate. Corresponding HRP-labeled secondary antibodies were used at the concentration of 1:10,000. Membranes were developed using SuperSignal West Pico chemiluminescent substrate kit (Thermo Scientific, Rockford, IL). Each sample has been probed in duplicate. Results were analyzed using ImageJ software (<https://imagej.nih.gov/>).

### **Quantitative PCR**

The technique was applied as described previously (Zabirnyk et al., 2010). Total RNA was isolated using RNeasy Kit (Qiagen, Valencia, CA) and complementary DNA was synthesized using SuperScript<sup>TM</sup> III First-Strand Synthesis SuperMix (Invitrogen, Carlsbad, CA). Expression of the gene of interest was measured by TaqMan quantitative PCR using

Roche LC480 cycler (Roche Diagnostics Corporation, Indianapolis, IN). TaqMan probes labeled with a specific fluorophore and primers for genes of interest were both designed using the [www.universalprobelibrary.com](http://www.universalprobelibrary.com) website against the cDNA sequences of *Papio anubis* and bought from IDT (IDT, Newark, NJ). The cDNA was pre-amplified using TaqMan PreAmp Master Mix Kit (Applied Biosystems, Foster City, CA). Kappa probe fast qPCR Kit Master mix (2x) Universal (Kappa Biosystems, Boston, MA) was used to run the Real-Time PCR. Each primer pair was validated for an efficiency of around 100%. The primer pairs and probes were as follows:

CB1: F ctggtgctgtgtcatcct, R aggctccaatgaagtgatag, probe 75

CB2: F ctctgtggtagcctgctg, R tttgtaggagggtggtagc, probe 13

Beta-actin: F attggcaatgagcggtc, R tcaggacaccgtaggtgc, probe 11.

The samples were run in duplicate for both target and reference (beta-actin) genes. The results were analyzed using Light Cycler 480 software (Roche Diagnostics Corporation, Indianapolis, IN).

## Chemicals

AEA, Tocrisolve, AM251, and AM630 were purchased from Tocris Bioscience (Bristol, United Kingdom). Ethanol (ultrapure, 200 proof) was purchased from American Bioanalytical (Natick, MA). To minimize the contact time between ethanol and humid air that could result in ethanol contamination with water, ethanol was aliquoted into smaller vials upon arrival. Each aliquot was used quickly, and vials were closed immediately after a dosage of ethanol was retrieved. All storage and handling of ethanol was performed per manufacturer's recommendations. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

## Statistics

Final plotting, fitting, and statistical analysis of the data were conducted using Origin 8.5.1 (OriginLab) and InStat 3.0 (GraphPad). Due to the nature of our study (e.g., the use of non-human primates), sample sizes in our work are small (does not exceed 4). Statistical analysis was conducted on groups of data points using either paired or unpaired *t* test, as *t* test is sensitive for data sets with limited sample sizes (de Winter, 2013).  $p < 0.05$  was considered statistically significant. Data are expressed as the mean  $\pm$  S.E.M., and  $n/N$  = number of measurements/number of fetuses. Due to the small sample sizes, in some instances, our study is underpowered to detect subtle differences between the groups.

## Results

### Alcohol gastric infusion in a baboon model of mid-pregnancy

Four baboons underwent alcohol gastric administration (1.8 g/kg) at 90, 100, and 110 days of gestation (Fig. 1A), following the general layout of a previously published protocol (Kochunov et al., 2010) with some modifications, as detailed in Material and Methods. Considering that the total length of pregnancy in baboons is 180 days (that is, about two-thirds of the length of pregnancy in humans), the selected timing of drinking procedures

corresponds to the second trimester of pregnancy in humans (Stevens, 1997). Upon alcohol infusion, maternal blood alcohol concentration reached approximately 80 mg/dL within 90 min (Fig. 1B). This pattern of drinking meets the definition of binge alcohol consumption, i.e., a pattern of drinking that results in a BAC of 80 mg/dL (<https://www.niaaa.nih.gov/alcohol-health/overview-alcohol-consumption/moderate-binge-drinking>). In our experimental model, blood alcohol concentration remained at 80 mg/dL for up to 180 min following the drinking episode (Fig. 1B). For comparison, the control group of animals (3 baboons) received orange-flavored infusion fluid that was isocaloric to the alcohol-containing solution. Alcohol concentration in the blood of animals receiving control infusion was undetectable (Fig. 1B). Mass spectroscopy data from maternal blood serum failed to detect a significant difference in circulating anandamide (AEA) and 2-arachydonoylglycerol (2-AG) between control- and alcohol-infused groups.

### Effect of fetal alcohol exposure on middle cerebral artery (MCA) function *in vivo*

Peak systolic velocity (PSV) of fetal MCA at 120 min following each alcohol exposure episode was significantly diminished when compared to pre-exposure levels as revealed by Doppler sonography ( $p < 0.01$ ) (Fig. 2A & B). As part of normal fetal development, PSV slightly increased during the course of the second trimester (Fig. 2B). Although there seemed to be a trend toward an increased fetal cerebral artery PSV in the alcohol-exposed group when compared to controls, this increase in PSV did not reach statistical significance ( $p > 0.05$ ) (Fig. 2B, right panel). Finally, alcohol exposure did not affect the resistive index (RI) of fetal MCA (Fig. 2C). Furthermore, RI remained unchanged at 120 days of gestation. Overall, the reduced PSV of fetal MCA at 120 min following each alcohol exposure episode is consistent with the possibility that alcohol administration to the mothers during the second trimester of pregnancy causes dilation of fetal MCA.

### Effect of fetal alcohol exposure on MCA function *in vitro*

To test the hypothesis that ethanol exposure dilates fetal cerebral arteries, and to eventually begin the exploration of the cellular mechanisms contributing to this alcohol action, we switched to an *in vitro* model. At  $120 \pm 5$  days of gestation, fetuses were harvested via C-section. Fetal middle cerebral arteries were dissected out and pressurized at 30 mmHg. At this time point during pregnancy, fetal cerebral arteries were consistently able to develop myogenic tone in response to 30 mmHg intravascular pressure. However, higher pressures sometimes resulted in arteries that failed to develop tone or respond to depolarization (see below). We did not find significant differences between fetal arteries from control- vs. alcohol-exposed fetuses for the following parameters: time to tone development, degree of myogenic tone, and arterial constriction in response to high (60 mM) KCl solution, an indicator of depolarization-induced constriction. However, ethanol (63 mg/dL; i.e., average concentration detected in amniotic fluid following maternal drinking) *in vitro* exposure for 10–15 min caused increases in diameter over pre-ethanol values in fetal arteries from both control- and alcohol-exposed fetuses: an average of 3.7 and 5.9%, respectively (Fig. 3A & B). Because diameter is linked to fluid flow by a power of 4 (Poiseuille's law), ethanol-induced dilation of fetal cerebral arteries would result in ~16 and ~26% increase in blood flow for control- and alcohol-exposed fetuses, respectively. Thus, this ethanol-induced dilation of cerebral arteries *in vitro* is consistent with the decreased PSV observed *in vivo*



and strongly suggests that the former is largely determined by vasodilation. More important, the fact that ethanol dilates *isolated* fetal cerebral artery segments under continuous perfusion indicates that this ethanol action does not require systemic metabolism (e.g., in liver) of alcohol, vasoactive factors from the systemic circulation, or intact autonomic regulation of arterial function. Rather, the molecular effectors of ethanol action are located within the fetal cerebral artery itself.

### **The role of cannabinoid receptors in ethanol-induced dilation of pressurized fetal cerebral arteries**

To address possible subcellular mechanisms mediating ethanol-induced dilation of fetal arteries, we focused on the eCBS. First, eCB signaling has been implicated in the development of ethanol-induced neurodegeneration in central neurons and FASD (Basavarajappa, 2015). Second, eCBS have been reported to regulate vascular diameter both *in vitro* and *in vivo* (Stanley & O'Sullivan, 2014). Third, a drop in brain perfusion may lead to cognitive dysfunction that resembles one observed with FASD (Amemiya et al., 2016). To begin to explore the involvement of the eCB's in ethanol-induced dilation of fetal cerebral arteries, we first used pharmacological means to demonstrate any possible involvement of cannabinoid receptors in alcohol action. Indeed, ethanol-induced *in vitro* dilation of pressurized fetal cerebral arteries was completely prevented by the mixture of AM251 and AM630, which are antagonists of cannabinoid receptors CB1 and CB2, respectively (Fig. 3B). Next, we used selective pharmacological ligands (agonists and antagonists) to assess the functional contribution of endocannabinoid receptors CB1 and CB2 to the regulation of fetal cerebral artery diameter and its modulation by alcohol. Selective block of CB1 receptor with AM251 resulted in fetal cerebral artery constriction, which was similar in cerebral arteries from control- vs. alcohol-exposed fetuses (Fig. 4A). To assess CB2 receptor function, we perfused fetal cerebral arteries with the CB receptor agonist AEA. AEA was tested in the presence of AM251 (to block any possible effect of AEA via CB1 receptors) on arteries that were pre-constricted with high potassium (60 mM KCl) solution. Soybean oil-based vehicle Tocrisolve™ was used to deliver AEA to cerebral arteries. Thus, AEA-free Tocrisolve™ served as control for AEA effects. In cerebral arteries from control-exposed fetuses, AEA (sub μM) failed to evoke dilation when compared to Tocrisolve™ alone (Fig. 4B). Remarkably, AEA caused a concentration-dependent ( $EC_{50} \approx 100$  nM) dilation in arteries from alcohol-exposed fetuses, in spite of the constriction evoked by the solvent (Fig. 4B). Moreover, AEA-induced dilation of fetal cerebral arteries disappeared when AM251 was complemented with the CB2 receptor blocker AM630 (Fig. 4C). Although highly consistent (observed in three different arterial segments), this outcome must be interpreted with some caution, as the three segments were isolated from the same donor. Collectively, our data indicate that ethanol-induced *in vitro* dilation involves CB receptors, and strongly suggest that fetal cerebral artery CB2 receptor function is enhanced by alcohol exposure *in utero*.

The increase in CB2 receptor function that mediates enhanced AEA-induced dilation of cerebral arteries in alcohol-exposed fetuses may be driven by several non-mutually exclusive mechanisms. They include increased gene expression or the receptor protein level, increased receptor affinity to the agonist, or functional changes driven by intracellular signaling

downstream of cannabinoid receptor activation. We first found that CB1 and CB2 mRNA transcripts did not differ in cerebral arteries from control vs. alcohol-exposed fetuses (Fig. 4D). In addition, protein levels probed by Western blot did not show statistically significant differences between cerebral arteries of control- vs. alcohol-exposed fetuses (Fig. 4E). These results indicate that the differences in transcription or translation of CB1 and CB2 cannot explain the functional changes observed in arteries from alcohol-exposed fetuses. Rather, these changes should be attributed to modifications of ligand-receptor affinity and/or modified downstream signaling (see Discussion).

## Discussion

In the current study, we identified for the first time that *in vivo* and *in vitro* exposure of fetal cerebral arteries from non-human primates (baboons) to ethanol at a period corresponding to the second trimester of human pregnancy evokes dilation of fetal cerebral arteries. This ethanol action is mediated by subcellular effectors located in the fetal cerebral artery itself: our study indicates that endocannabinoid receptors are the mediators of alcohol-induced dilation of fetal cerebral arteries. Moreover, based on a pharmacological approach with agonists and blockers of CB receptors, complemented with Western blotting and quantitative PCR, our study advances the idea that CB2 receptor function is increased in the cerebral arteries from fetuses exposed to alcohol *in utero*.

About a third of women who consume alcohol during pregnancy engage in binge drinking, i.e., drinking that results in a rapid rise of BAC reaching 80 mg/dL within 2 h (<http://www.cdc.gov/media/releases/2015/p0924-pregnant-alcohol.html>). Often, episodic moderate-to-heavy alcohol intake (such as during binge drinking) may produce much higher BACs (Diamond, 1991). As documented by numerous studies, a BAC at or above 80 mg/dL produces the most negative effects on the developing fetus (Maier & West, 2001; May & Gossage, 2011). Thus, it comes as no surprise that the FASD-affected births are most frequent in geographic areas with a high prevalence of alcohol binge drinking (May & Gossage, 2011; May et al., 2005; Viljoen et al., 2005). Importantly, our findings on the fetal cerebral circulation and eCBS were obtained in a model of alcohol exposure that rendered 80 mg/dL alcohol in maternal blood (Fig. 1).

Among all organs, the brain is the most sensitive to prenatal alcohol exposure, as the latter results in a wide range of structural and functional brain abnormalities (Nunez, Roussotte, & Sowell, 2011; Ramadoss, Lunde, Piña, Chen, & Cudd, 2007). Considering that the cerebral circulation provides key nutrients for effective neuronal function, the idea that alterations in fetal cerebral blood flow may accompany maternal alcohol consumption has been considered by several groups. Experimental work in sheep showed an attenuated adaptation of cerebral blood flow to neuronal damage in fetuses exposed to maternal binge drinking. This attenuated adaptation could not be explained by changes in vessel density but has been interpreted as arising from malfunctioning of cerebral blood vessels (Simon, Mondares, Born, & Gleason, 2008). Indeed, a recent study on a mouse model of pregnancy demonstrated that maternal binge drinking during the second trimester of pregnancy significantly reduced acceleration and the velocity time integral in fetal MCA (Bake, Tingling, & Miranda, 2012). Furthermore, our study demonstrates that alcohol

administration to non-human primates during mid-pregnancy leads to a decreased PSV, as determined by Doppler ultrasound (Fig. 2). Collectively, these findings seem to indicate that maternal binge alcohol exposure may cause dilation of fetal cerebral arteries. The latter poses a substantial threat to fetal development (Hernandez-Andrade, Serralde, & Cruz-Martinez, 2012). Indeed, cerebral vasodilation may cause a drop in perfusion pressure, likely resulting in cognitive deficiency and skull malformation in children and young adults (Gordon, 2009; Yoon, Parsa, & Horton, 2013). Remarkably, these symptoms are similar to major features of FASD (Nunez et al., 2011).

However, our *in vivo* findings should be interpreted with caution – in our experimental model, the effect of alcohol was observed in the presence of anesthesia (i.e., induction with ketamine followed by maintenance using isoflurane). In rodents, however, ethanol was shown to promote a similar increase in cerebral blood volume in awake rats when compared to isoflurane-anesthetized animals (Luo et al., 2007). Moreover, a recent study of fetal cerebrovascular function in a mouse model also employed ethanol administration under isoflurane anesthesia (Bake et al., 2012). Because the control group in our study received anesthesia identical to that administered to alcohol-receiving baboons, a likely explanation for our *in vivo* findings is that ethanol itself is responsible for the changes in hemodynamics of fetal cerebral circulation. However, we cannot rule out that the final alcohol effect may be conditioned by the presence of ketamine (Silvestre, Pallarés, Nadal, & Ferré, 2002), with this fact adding a layer of complexity to the interpretation of our *in vivo* data.

A very critical finding from the current study is the demonstration that ethanol is able to evoke vasodilation in isolated *in vitro* pressurized fetal cerebral arteries (Fig. 3) – we documented alcohol-induced fetal artery dilation in the absence of complex neuronal circuitry and vasoactive factors from systemic circulation. Our findings may help to explain previously reported human data that demonstrated an alcohol-induced decrease in metabolic activity in some regions of human brain (Volkow et al., 1988; Wang et al., 2000). It should be underscored, however, that studies on arteries pressurized *in vitro* have their limitations. In particular, we cannot exclude the possibility that the complex neuronal integrity that is present in the intact neurovascular unit, or maternal vasoactive circulating factors, contribute to the *in vivo* response of fetal cerebral arteries to ethanol administration. Identification of all possible factors is daunting, yet deserves a thorough consideration and future experimental testing.

Our work also revealed that alcohol-induced dilation of fetal arteries disappeared in the presence of CB receptor blockers (Fig. 3). To our knowledge, this finding is the first ever to identify specific mechanisms of vasoactive effects of maternal alcohol on *fetal* cerebral arteries. Outside the vascular system, eCB components are known to be targeted by alcohol. For example, the neuronal eCB signaling system represents an important target for alcohol, and pharmacological modulation of neuronal eCBS has been proposed as an emerging therapy for treatment of alcohol-use disorders (Pacher, Bátkai, & Kunos, 2006; Pava & Woodward, 2012; Serrano et al., 2012; Sidhpura & Parsons, 2011). With regard to episodic drinking (such as binge drinking), CB1 receptor-mediated agonism of the endocannabinoid system has been implicated in modulating binge-like alcohol intake in mouse models

(Agoglia, Holstein, Eastman, & Hodge, 2016; Linsenbardt & Boehm, 2009; Zhou, Huang, Lee, & Kreek, 2016).

In the current work, we failed to detect changes in transcript or protein levels of CB1 and CB2 receptors within cerebral arteries from alcohol-exposed fetuses when compared to control fetuses (Fig. 4D–E). However, alcohol exposure *in utero* enhanced fetal cerebral artery dilation in response to the pharmacological agonist AEA when CB1 receptor was selectively blocked by AM251. The increased response of the CB2 receptor to constantly perfused AEA in the absence of increased CB2 protein level strongly suggests an enhanced function of CB2 receptors. This enhanced functionality may result from several not mutually exclusive pathways, such as increased affinity of CB2 receptor to AEA, enhanced efficiency of translating AEA binding into the receptor activation, and increased downstream signaling effectors of CB2 activation. The possible role of endocannabinoid production within the fetal cerebral artery and its modulation by the exogenously introduced pharmacological agents should also be explored. These studies are currently under way.

Noteworthy, the CB2 receptor's upregulation was shown to contribute to the delayed neuroprotective effect against cerebral ischemia induced by MCA occlusion in rats (Ma et al., 2011). It is tempting to speculate that enhanced CB2 receptor function observed in cerebral arteries of alcohol-exposed fetuses might represent a protective mechanism against a repeated drop in fetal artery PSV in the presence of alcohol.

In synthesis, our study presents the first robust evidence that the key components of the fetal cerebral artery eCBS (CB2) are targeted by alcohol exposure *in utero*, underscoring that elements of this system, the EC receptors in particular, are major determinants of the vasodilatory effect of alcohol on fetal cerebral arteries. These findings may pave the road to a better understanding of the pathophysiology of FAS/FASD as a result of hemodynamic dysregulation in the fetal brain, as well as to open up a potential therapeutic application of CB receptor inverse agonists (such as rimonabant) as a rescue therapy to normalize fetal cerebral circulation caused by maternal alcohol consumption. Rimonabant blocks psychoactive effects and several cardiovascular effects of  $\Delta^9$ -tetrahydrocannabinol (THC) in humans (Huestis et al., 2001). Interestingly, our data show that ethanol dilation of fetal cerebral arteries is totally blunted by the combination of AM251 and AM630, which are both structurally close to rimonabant. Further *in vivo* studies are imperative to develop endocannabinoid antagonists/inverse agonists with no major side effects to consider their potential use in ameliorating negative consequences of maternal alcohol consumption that involves disruption of fetal arterial function.

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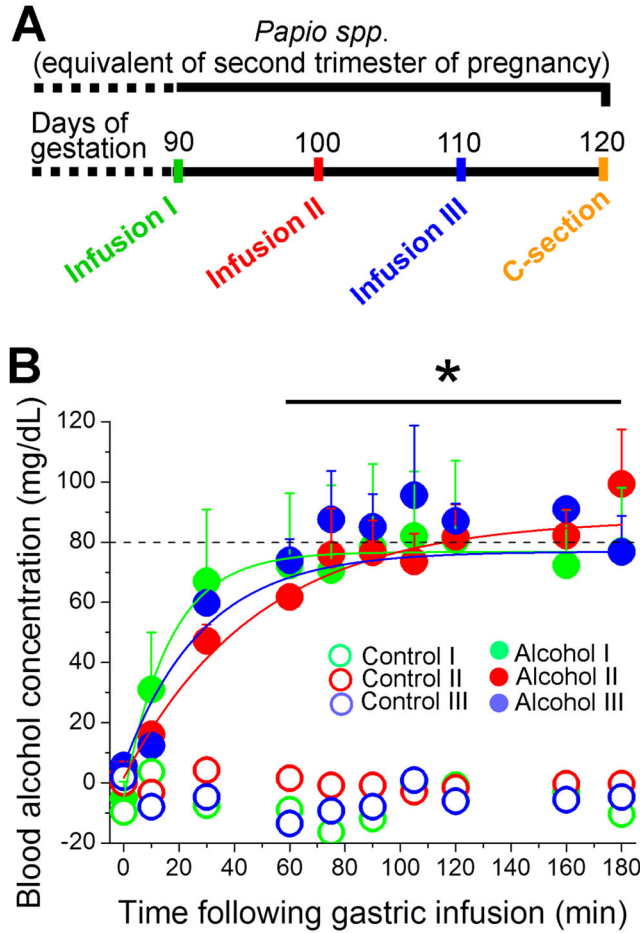
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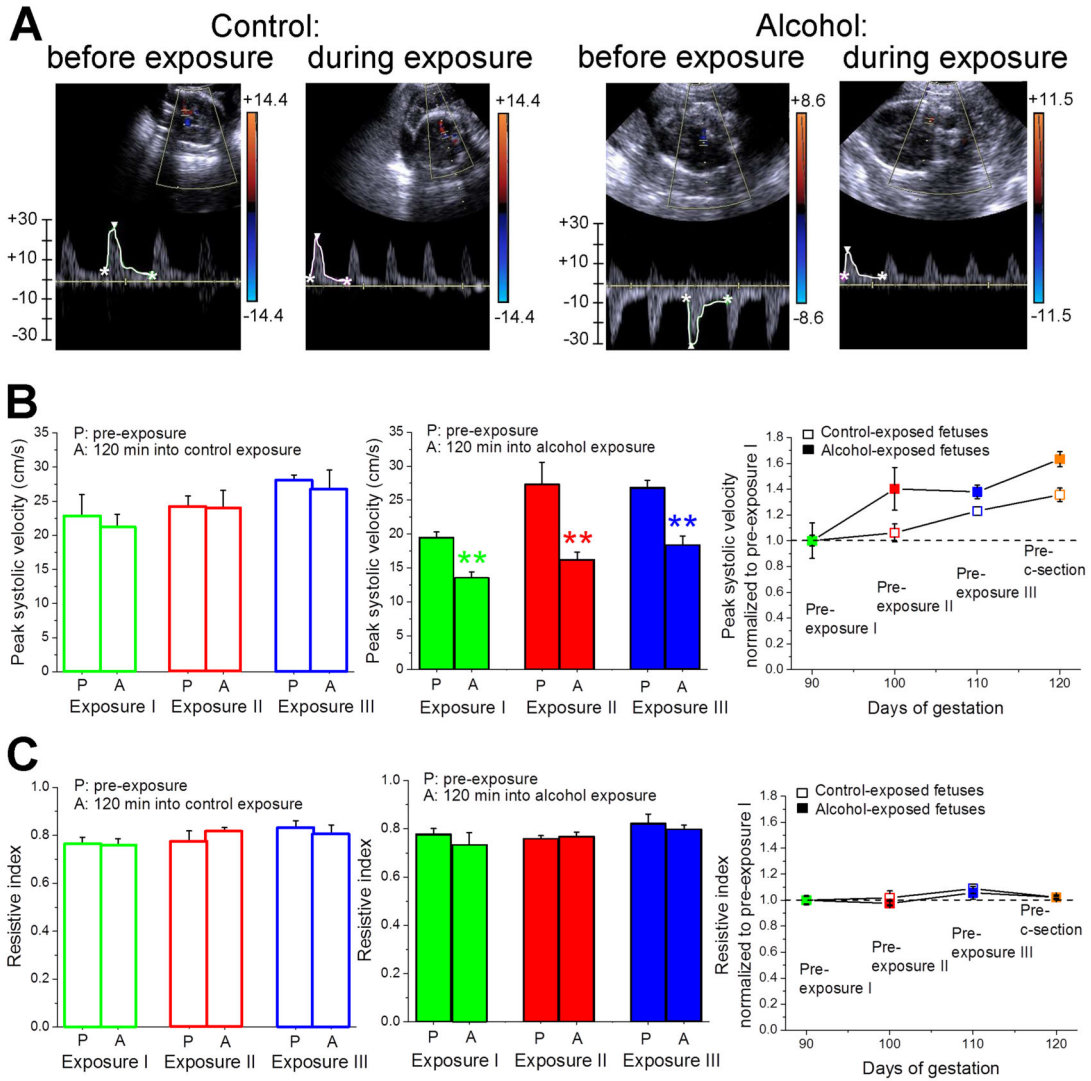
**Highlights**

- Mechanisms of fetal brain damage by alcohol are largely unclear.
- Baboons were used to study alcohol's effect on fetal cerebral arteries.
- Alcohol dilates fetal cerebral arteries via cannabinoid receptors.
- Alcohol exposure enhances cerebral artery CB2 receptor responses to anandamide.
- Alcohol exposure does not change CB1 and CB2 mRNA or protein levels.



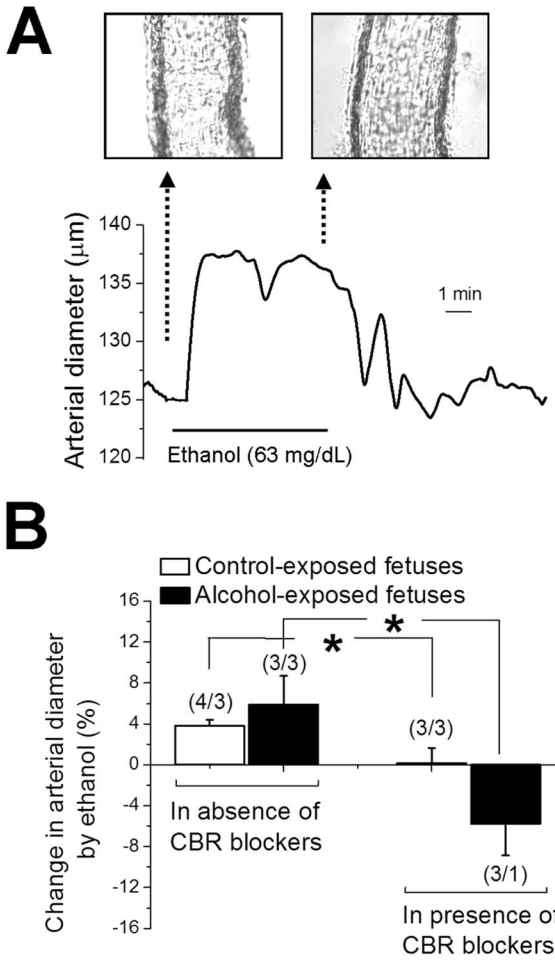


**Fig. 1. Alcohol gastric infusion in a baboon model of pregnancy**  
**A.** Schematic representation of experimental design. Each pregnant baboon underwent three infusion episodes within the human equivalent of the 2nd trimester of pregnancy. Fetal cerebral arteries were harvested following Cesarean section at 120 days of baboon gestation.  
**B.** Alcohol level in maternal blood as a function of time following gastric infusion of alcohol-containing fluid (N = 4). Animals receiving control fluid did not have detectable amounts of alcohol in the blood (for visual clarity, data points from only one out of three baboons in the control-infused group are shown). Data at each time point in alcohol- versus control-receiving groups were compared using independent unpaired *t* tests. \*Significantly different from blood alcohol level in control-receiving group, applicable to each alcohol-/control-receiving episode, *p* < 0.05.

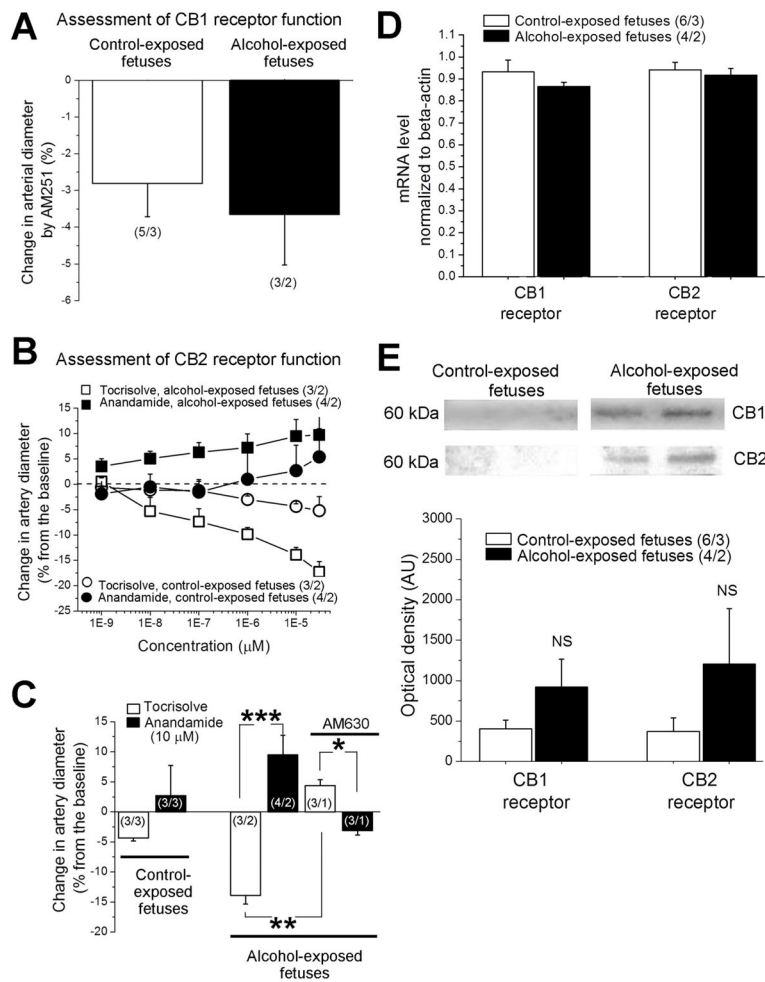


**Fig. 2. Doppler sonography evaluation of middle cerebral artery (MCA) function following maternal gastric infusion of either control- or alcohol-containing fluid**

**A.** Snapshots showing fetal MCA blood flow velocity before and 120 min following maternal gastric infusion of control (two left panels) vs. alcohol-containing fluid (two right panels). **B.** Averaged data showing a statistically significant drop in fetal cerebral artery peak systolic velocity following maternal gastric infusion of alcohol-containing but not control fluid. Maternal alcohol infusion does not affect gestational age-dependent increase in peak systolic velocity (panel on the right). In **B** and **C**, data at each exposure were compared using independent paired *t* tests. \*\*Significantly different from pre-exposure level in corresponding exposure episode, *p* < 0.01. **C.** Averaged data showing lack of statistically significant difference in resistive index of fetal cerebral arteries from control- vs. alcohol-infused mothers. Control-infused: N = 3; alcohol-infused: N = 4.



**Fig. 3. Ethanol-induced dilation of *in vitro* pressurized fetal MCA**  
**A.** Trace showing fetal cerebral artery dilation in response to 63-mg/dL ethanol. **B.** Bar graph displays averaged data: ethanol-induced dilation of fetal cerebral artery vanishes in presence of CB1 and CB2 receptor blockers AM251 and AM630, respectively. Data from control- and alcohol-exposed fetuses were compared using independent unpaired *t* tests. \*Significantly different from data in absence of CBR blockers, *p* < 0.05. (n/N) = number of artery segments/number of fetuses.



**Fig. 4. Evaluation of CB1 and CB2 receptor function in pressurized fetal cerebral arteries *in vitro***

**A.** Averaged data showing similar degree of artery constriction in response to CB1 receptor blocker AM251; data were compared using unpaired *t* test. **B.** Concentration-response curves for anandamide in vehicle Tocrisolve™ and corresponding curves for anandamide-free Tocrisolve™ obtained from cerebral arteries of control- vs. alcohol-exposed fetuses in presence of AM251 and pre-constricted with KCl. **C.** Averaged data reflecting anandamide-induced cerebral dilation in arteries from alcohol-exposed fetuses; this dilation disappears when AM251 is complemented with CB2 receptor blocker AM630. Artery data from control-exposed fetuses were compared using unpaired *t* test. For alcohol-exposed fetuses, three sets of data were compared using independent unpaired *t* tests: Tocrisolve- versus anandamide-induced changes in artery diameter in absence and in presence of AM630, and Tocrisolve-induced changes in artery diameter in absence versus presence of AM630. \*Significantly different from Tocrisolve-induced changes in diameter, *p* < 0.05. \*\*Significantly different from Tocrisolve effect, *p* < 0.01. \*\*\*Significantly different from Tocrisolve effect in absence of AM630, *p* < 0.001. (n/N) = number of artery segments/ number of fetuses. **D.** Averaged values of mRNA concentration normalized to beta-actin show no significant differences in gene expression of CB1 and CB2 receptors between

control- and alcohol-exposed fetuses. In **D** and **E**, data for CB1 and CB2 receptors were compared using independent unpaired *t* tests. **E**. Representative images of Western blot data (top panels) in cerebral arteries from control- and alcohol-exposed fetuses. Artery homogenate samples contained equal amount of total protein. Bar graph shows averaged values of optical density corresponding to CB1 and CB2 protein levels. NS: not significant.