Committee for Aid and Education in Neurochemistry (CAEN)
CATEGORY 1B: Research supplies for use in the applicant's home laboratory – ROUND DATE DECEMBER 2015.

Name: Joice Stipursky Silva, PhD.
Federal University of Rio de Janeiro (UFRJ)
Institute of Biomedical Sciences (ICB)
Laboratory of Cellular Neurobiology
Email address: joice@icb.ufrj.br
Current Position: Assistant Professor

Project Background

EFFECT OF ETHANOL EXPOSURE DURING PREGNANCY ON ENDOTHELIAL CELLS FUNCTION IN THE DEVELOPING BRAIN: IMPACTS ON SYNAPTOGENESIS.

Fetal alcohol syndrome (FAS) refers to the group of anomalies affecting children due to alchool intake during gestational period. FAS is the most severe form of the conditions included in the Fetal alcohol spectrum disorders (FASD). Recent reports demonstrate that FAS is believed to occur in between 0.2 and 9 per 1000 live births in the United States. FAS affected individuals present clinical features such as facial anomalies, short height, low body weight, and prevalent cognitive and behavioral impairments due to disturbance in central nervous system (CNS) development (CDC, 2015; May et. al., 2009; May et. al., 2014).

The interface between CNS and circulatory system is constituted by the blood brain barrier (BBB) structure, that allows transference of nutrients, O2, hormones and other molecules, from the blood stream to the brain parenchyma, and prevent the free passage of potentially toxic molecules to the brain tissue. The BBB is formed during early embryonic developmental stage by angiogenic sprouts derived from blood vessels that invade the neural tube from exogenous regions. During blood vessel growth, angiogenic branches interacts with adjacent neural stem cells, astrocytes and neurons, to form the neurovascular unit of the brain (Nakagawa et al., 2009; Takahashi et al., 2015; Walchli et al., 2015, **Siqueira et al, 2017**).

Synaptic transmission constitute the base of information transference events by neurons in the CNS (Diniz et al., 2012; Shen & Cowan 2010). Several alterations induced by ethanol on neurotransmiters receptors such as NMDA and GABA receptors subunits, were pointed as crutial to disturb synatogenesis and synaptic plasticity, negatively impacting memory acquisition and consolidation (Zorumski et al., 2014, Lovinger et al., 2013). In the brain of individuals exposed to ethanol during pregnancy several neurotransmission systems are disturbed, resulting in inbalance of the excitatory and inhibitory transmission (Olney, 2004). Interestingly, synaptogenesis and synaptic plasticity events are also modulated by molecules tipically related to vascular growth, such as the

VEGF (vascular endothelial growth factor) and Angiopoietin-1 (Kosacka et al., 2006; Tilo et al., 2012, Guérit et al., 2014).

Although ethanol exposure, generally promotes deleterious damage on endothelial cells of the adult brain blood vessels, as well as directly affects neuronal function and synaptic transmition, resulting in the severe cognitive and behavioral deficits observed in FAS individuals (Williams et. al, 2015), little is known about endothelial dysfunction as a mediator of neuronal synaptogenesis deficits during brain development.

Although the vascular system is one of the first targets of Ethanol, after it's intake, little is known about the methabolic and molecular alterations on endothelial cells of the BBB, that could impact the integrity of the nascent neuronal networks. It is possible that the nascent vascular tree in the embryo brain, early in development, can be targets of Ethanol exposure, directly impacting neuronal generation, maturation and synapse formation. **Therefore, it is essential to understand the vascular-neuronal interactions and to create strategies that can promote vascular integrity recovery and maintenance, to ensure the protection of neuronal populations and synaptic connections to ease the cognitive deficits of individuals with FAS.**

In this project we are investigating the deleterious effect of ethanol exposure during pregnancy on endothelial cells of the developing cerebral cortex, and the impact of the endothelial dysfunction on the formation and maturation of neuronal synapses.

1) HYPOTHESIS TESTED

We tested whether ethanol exposure during pregnancy impairs angiogenesis during BBB and neuronal development.

Specificaly, we will:

1.1) Evaluate ethanol induced dysfunction on endothelial cells: quantification of endothelial tight junction structures, glucose transporter expression, ethanol metabolizing enzime machinery expression and oxidative stress induction with generation of oxigen and nitrogen reactive species (ROS/RNS);

1.2) Evaluate whether endothelial dysfunction impact neuronal synapses formation and maturation: quantification of neuron numbers and presynaptic and post synaptic protein distribution in neuronal cultures, after exposure of these cultures with endothelial cells condicioned medium.

2) OUTCOMES

2.1) Characterization of endothelial cells dysfunctions induced by Ethanol exposure *in vitro*.

2.1.1) Tight junction protein ZO-1 presents reduced levels in endothelial cells exposed to ethanol.

Human brain microcapillary endothelial cells lineage (HBMEC) were analysed by immunocytochemistry and shows ZO-1 distribution cell-cell membrane contacts and surface (Fig.1A), which was less evident after Ethanol treatment (Fig.1 B). Tight junction organization levels (TiJOR, Fig. 1C) suggest that Ethanol reduces adhesion and junction stability, as well as reduces ZO-1 protein levels (Fig. 1D). N=3

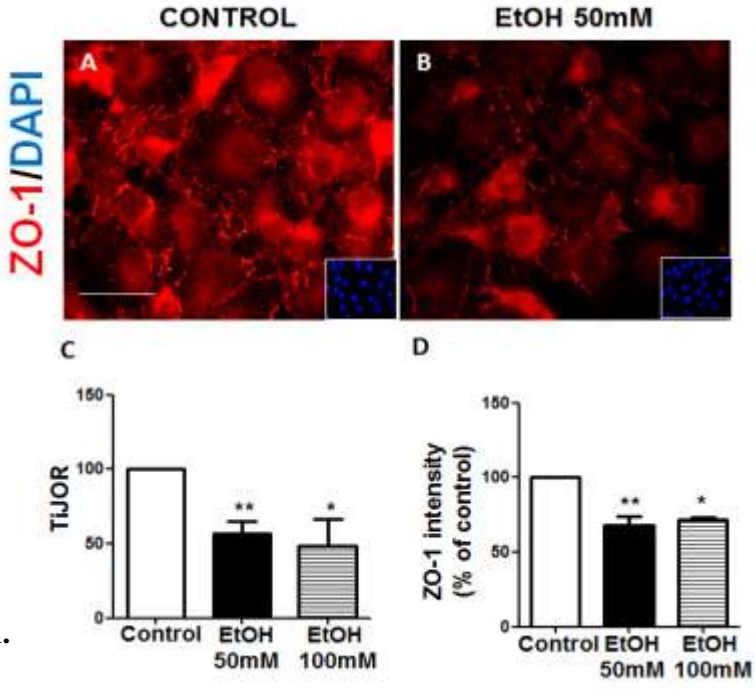

Figure 1.

2.1.2) Ethano reduces Glucose transporter GLUT-1 levels in endothelial cells.

GLUT-1 was analysed by immunocytochemistry and in cell western, and we observed decreased distribution on HMBEC membranes (Fig. 2A-B) and levels of this protein (Fig2. C, D) after Ethanol treatment. N=4

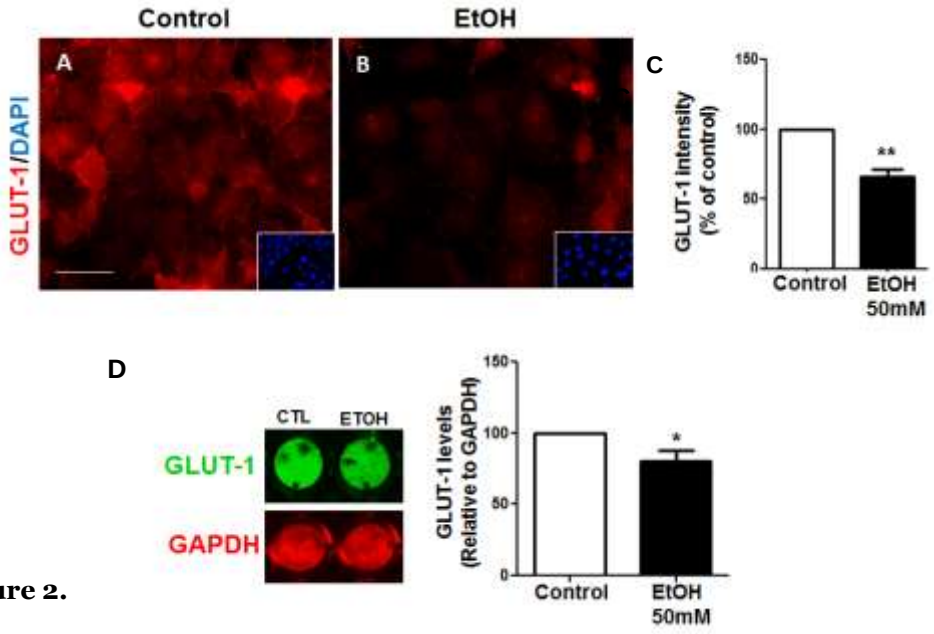

Figure 2.

2.1.3) Catalase levels are increased in endothelial cells exposed to Ethanol.

Catalase enzyme levels were analysed by immunocytochemistry, and we observed that Ethanol increases distribution and levels on HMBEC membranes (Fig. 3 B, D) and higher concentrations of Ethanol reduces the levels of this enzyme (Fig2. C, D)after Ethanol treatment. N=3

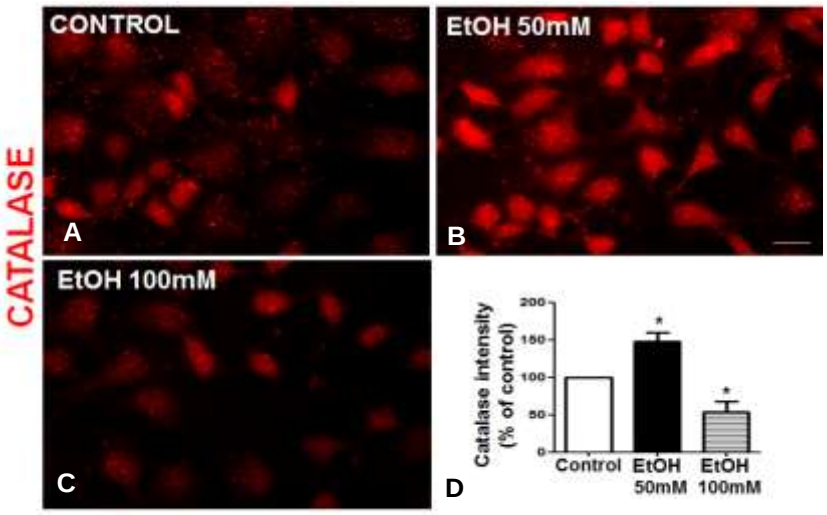

Figure 3.

2.2) Evaluation of the impacts of Ethanol-induced endothelial dysfunction on neuronal maturation and synaptogenesis *in vitro*.

2.2.1) Soluble factos secreted by endothelial cells exposed to Ethanol decreases neurons numbers.

HMBEC cells were treated or not with Ethanol (50mM) for 2hs and after this period cells were washed and a fresh medium as added. After 24hs culture medium as colected and used as Endothelial conditioned medium (CM-EC and CM-EC-EtOH). Neurons were isolated from embryonic (E15.5) cerebral cortex and cultivated for 12days. After this period neurons were tretated with CM-EC or CM-EC-EtOH for 3 hours. We observed that CM-EC-EtOH reduced the numbers of β-tubulinIII positive neurons in these cultures (Fig. 4 C, E), withtout affect total cell numbers (Fig. 4D) when we compare with control or CM-EC conditions. N=3.

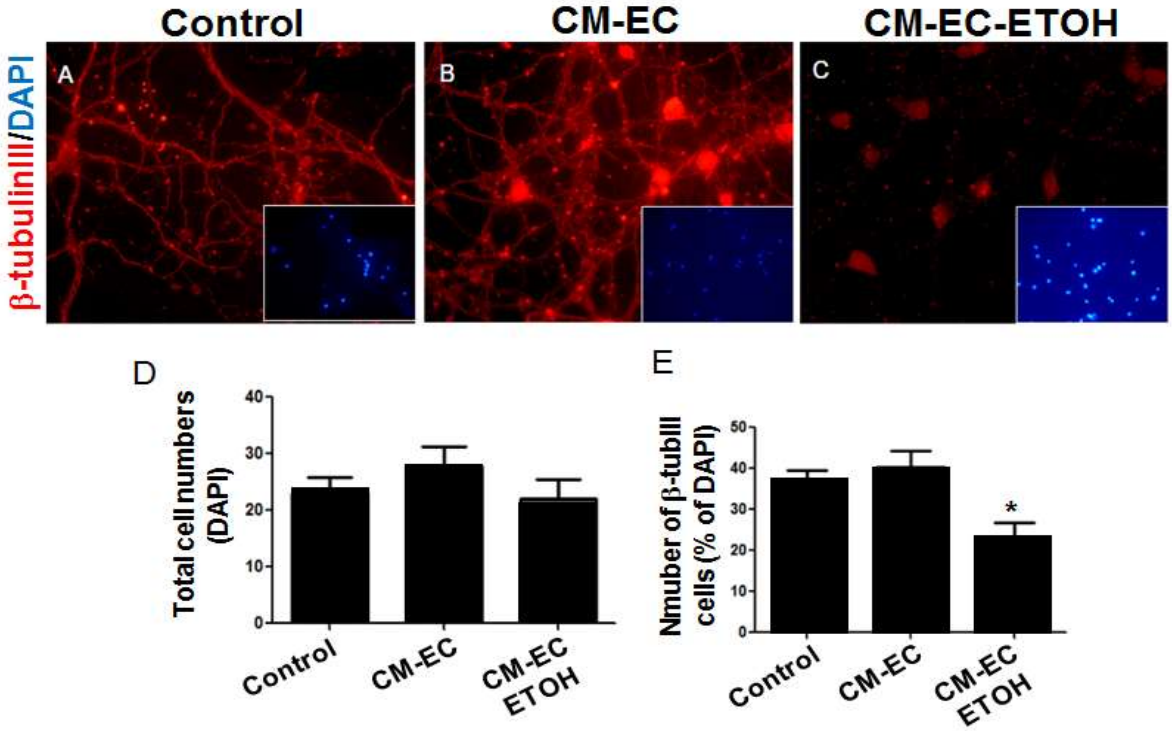

Figure 4.

2.2.2) Soluble factos secreted by endothelial cells exposed to ethanol inhibits synapse formation.

In the same context described above (item 2.2.1), our preliminary results suggest that, interestingly, CM-EC induces synapse puncta formation, labeled for Synaptophysin (pre synaptic terminal) and PSD-95 (post synaptic terminal) (Fig. 5 B, D). However CM-EC-EtOH drasticaly reduces the numbers of synaptic puncta along neuronal processes (Fig. 5 C, D), when we compare with control condition (Fig.5 A). N=2.

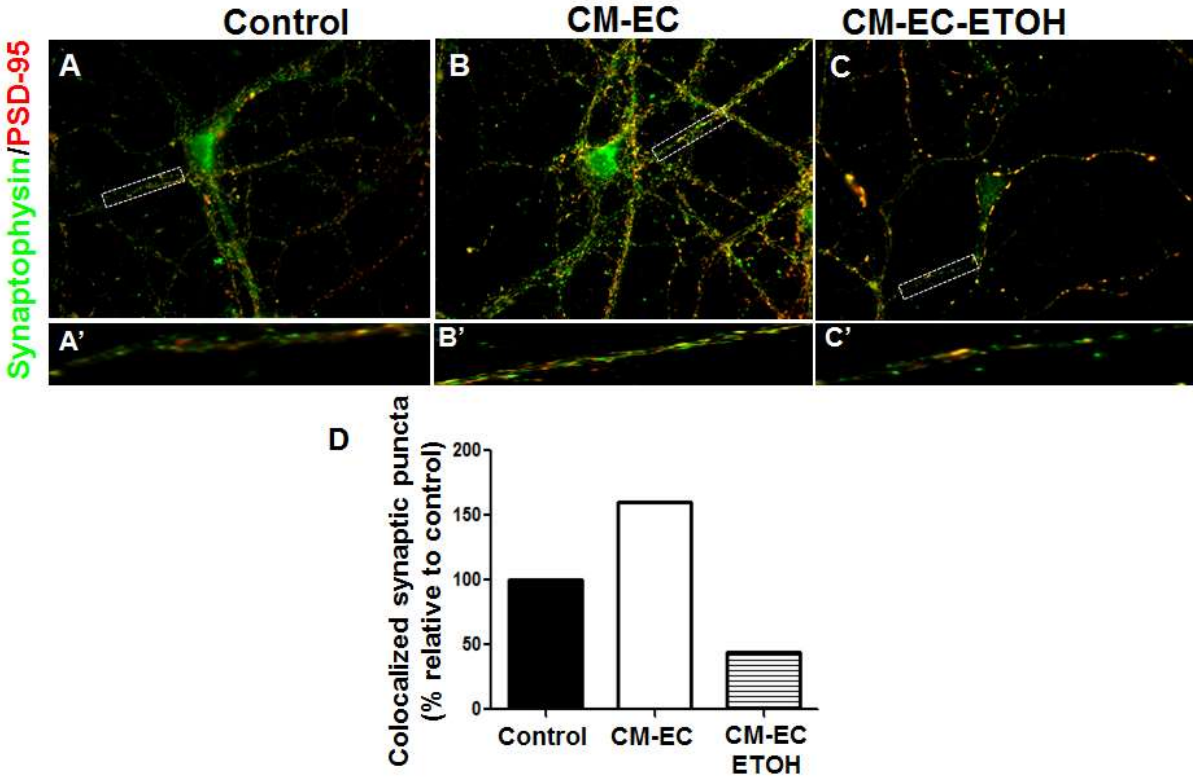

Figure 5.

3) PRELIMINARY CONCLUSIONS

Our preliminary data demonstrates that in fact Endothelial cells are direct targets of ethanol effects *in vitro*, and possibly blood vessels *in vivo*. Specificaly we obseved that ethanol negatively impacts tight junction protein ZO-1 expression and distribution, possibly inducing less adhesion between endothelial cell monolayer, which would induce blood vessel leakage *in vivo*. Another important aspect is that GLUT-1 levels are reduced in endothelial cells, suggesting that ethanol might decrease endothelial cell capacity to transport glucose across BBB into the brain parenchyma, which would in turn impair energy supply to CNS. In accordance with these negative impacts on endothelial cells function, Catalase levels are increased after exposure of ethanol in the concentration that induced many of the deleterious effects described above (50mM), suggesting that endothelial cells might increase Catalase expression as a compensatory mechanisms to minimize ethanol toxic effects. However, in higher concentration of ethanol (100mM), Catalase levels decreases, suggesting that this concentration might trigger more agressive effects in endothelial funcions.

In our investigation of the repercussion of endothelial dysfunction on neuronal development, we observed that normal endothelial cells secrete factors that, although does not affect neuronal numbers, increases neuronal maturation by induction of synapse formation. However, endothelial cells previously exposed to ethanol, possibly secretes a different repertoire of secreted factors, that, on the other hand, impairs synapse formation.

Together these preliminary data suggest that, by the fact that in normal condition endothelial cells might be inductors of synapse formation and maintenance, damage induction by ethanol exposure on these cells might switch the role of these cells into a "cytotoxic" profile that impairs neuronal maturation and synapse formation.

4) ANALYZES IN PROGRESS

Currently we are performing the additional experiments:

3.1) Synapse formation after MC-EC-EtOH exposure;

3.2) Evaluation of ROS/RNS production by endothelial cells exposed to ethanol, to describe the oxidative stress induction profile in this condition;

3.3) Catalase activity measurements in endothelial cells exposed to EtOH;

3.4) *In vivo* ethanol exposure in pregnant mice, to investigate blood vessel and endothelial maturation and function in early periods of brain development, and synaptogenesis in post-natal period.

5) TEAM INVOLVED IN THE PROJECT

Joice Stipursky, PhD, Assistant Professor, Institute of Biomedical Sciences, UFRJ;
Michele Siqueira, Master Student, Post-graduation Program in Morphological Sciences, Institute of Biomedical Sciences, UFRJ;
Diego Gisbert, Undergrad Student, Medical School, Universidade Estácio de Sá;
Anne Caroline Leopoldo, Undergrad Student, Biological Sciences, UFRJ;
Alexandre Gomes, Undergrad Student, Biological Sciences, UERJ.

6) FINANCIAL REPORT

PRODUCT	COMPANY	PRICE/UNIT (USD)	QUANTITY	FINAL PRICE (USD)
Cellular ROS/RNS Detection Assay Kit (Abcam)	Abcam	345	1	345
Mouse anti-Synaptophysin	Abcam	429	1	429
Rabbit anti-PSD-95 antibody	Abcam	425	1	425
Rabbit anti-GLUT1 antibody	Abcam	425	1	425
Rabbit anti-ZO-1 antibody	Thermo fisher	414	1	414
Catalase antibody	Abcam	425	1	425
199 medium	Sigma aldrich	80	4	320
Ethanol Assay Kit	Abcam	539	1	539
B27 supplement, 10ml	Gibco	160	5	800
Goat anti-Rabbit IgG (H+L) Secondary Antibody, DyLight 488	Thermo fisher	220	1	220

Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, DyLight 594	**Thermo fisher**	**210**	**1**	**210**
Neurobasal culture medium, 500 mL	**Gibco**	**100**	**5**	**500**
			TOTAL	**5052**

7) Acknowledgement

I would like to thank ISN for the financial support that became possible to perform the initial steps of this project.

8) REFERENCES

Centers for Disease Control and Prevention (CDC). Fetal alcohol syndrome-Alaska, Arizona, Colorado, and New York, 1995-1997. MMWR Morb Mortal Wkly Rep. 51(20):433-5. 2002.

Data & Statistics Prevalence of FASDs". *Center for Disease Control and Prevention*. April 16, 2015. Retrieved 10 June 2015.

Diniz LP, Almeida JC, Tortelli V, Lopes CV, **Stipursky J**, Perdigão P, Kahn S, Miranda J, Castro N, PanizzuttiM RA & Gomes FCA. Astrocyte-induced synapses are mediated by transforming growth factor beta 1 through modulation of D-serine levels in the cerebral cortex. J Biol Chem. 30;287(49):41432-45.

Guérit S[1], Allain AE, Léon C, Cazenave W, Ferrara N, Branchereau P, Bikfalvi A. VEGF modulates synaptic activity in the developing spinal cord. Dev Neurobiol. 2014 Nov;74(11):1110-22. doi: 10.1002/dneu.22187. Epub 2014 May 24.

Kosacka J[1], Nowicki M, Kacza J, Borlak J, Engele J, Spanel-Borowski K. Adipocyte-derived angiopoietin-1 supports neurite outgrowth and synaptogenesis of sensory neurons. J Neurosci Res. 2006 May 15;83(7):1160-9.

Lovinger DM[1], Roberto M. Synaptic effects induced by alcohol. Curr Top Behav Neurosci. 2013;13:31-86. doi: 10.1007/7854_2011_143.

May PA, Baete A, Russo J, Elliott AJ, Blankenship J, Kalberg WO, Buckley D, Brooks M, Hasken J, Abdul-Rahman O, Adam MP, Robinson LK, Manning M, Hoyme HE. Prevalence and characteristics of fetal alcohol spectrum disorders. Pediatrics. 134:855-66. 2014.

Nakagawa S, Deli M, Kawaguchi H, Shimizudani T, Shimono T, Kittel A, Tanaka K, Niwa M. A new blood brain barrier model using primary rat brain endothelialcells, pericytes and astrocytes. Neurochem Int. 54:254-263. 2009.

Olney JW. Fetal alcohol syndrome at the cellular level. Addict Biol. 2004 Jun;9(2):137-49; discussion 151.

Shen K[1], Cowan CW. Guidance molecules in synapse formation and plasticity. Cold Spring Harb Perspect Biol. 2010 Apr;2(4):a001842. doi: 10.1101/cshperspect.a001842.

Siqueira M. and Stipursky J. Radial glia secreted factors controls Angiogenesis in the developing cerebral cortex. Molecular Neurobiology, 2017 May 18. doi: 10.1007/s12035-017-0557-8.

Tillo M[1], Ruhrberg C, Mackenzie F. Emerging roles for semaphorins and VEGFs in synaptogenesis and synaptic plasticity. Cell Adh Migr. 2012 Nov-Dec;6(6):541-6. doi: 10.4161/cam.22408. Epub 2012 Oct 17.

Wälchli T[1], Mateos JM[2], Weinman O[3], Babic D[4], Regli L[5], Hoerstrup SP[6], Gerhardt H[7], Schwab ME[3], Vogel J[8]. Quantitative assessment of angiogenesis, perfused blood vessels and endothelial tip cells in the postnatal mouse brain. Nat Protoc. 2015 Jan;10(1):53-74. doi: 10.1038/nprot.2015.002.

Williams, J.F., Smith, V.C. & Committee On Substance, A. Fetal Alcohol Spectrum Disorders. Pediatrics 136, e1395-1406. 2015.

Zorumski CF[1], Mennerick S[2], Izumi Y[2]. Acute and chronic effects of ethanol on learning-related synaptic plasticity. Alcohol. 2014 Feb;48(1):1-17. doi: 10.1016/j.alcohol.2013.09.045. Epub 2013 Dec 16.