The Effect of Alcohol on Genetically Modified Exosome Deposition in the Hypothalamus

By

Brigette Ramos

A thesis submitted to the

School of Graduate Studies

Rutgers, The State University of New Jersey

In partial fulfillment of the requirements

For the degree of

Master of Science

Graduate Program in Physiology and Integrative Biology

Written under the direction of

Dipak Sarkar

And approved by

_____

_____

_____

_____

_____

New Brunswick, New Jersey

May 2020

ABSTRACT OF THE THESIS

The Effect of Alcohol on Genetically Modified

Exosome Deposition in the Hypothalamus

By BRIGETTE RAMOS

Thesis Director:

Dipak, Sarkar, Ph.D

Fetal Alcohol Syndrome (FAS) is a condition, particularly found in children, characterized by damage to the central nervous system as a result of alcohol exposure during a woman's pregnancy (Wilhoit et al., 2017). Areas of the brain that are affected include the hippocampus, basal ganglia, cerebellum, and hypothalamus. Once exposed to alcohol, the production of Beta-endorphins (βEP), a neuronal peptide involved in inhibiting stress in the hypothalamic arcuate nucleus, is reduced (Sprouse-Blum et al., 2010). On the other hand, there are cells, such as microglia, that can be stimulated during alcohol exposure. Microglia perform several essential functions in the brain, such as a role in neuroinflammation, regulation of brain development, and work as CNS macrophages (Colonna et al., 2017). Microglia are also capable of releasing exosomes, small membrane vesicles involved in neuronal communication and immune functions, including adaptive and innate immune responses (Fruhbeis et al., 2012).

Using a rat animal model, our overall objective revolved around how exosomes, derived from genetically modified microglial cells, could be introduced through the periphery to influence βEP and glial interaction in the brain, and whether transportation of these exosomes could be altered by alcohol administration. We observed that the exosomes successfully passed through the blood-brain barrier (BBB) and were able to deposit into the hypothalamic arcuate nucleus. There was an increase in exosome deposition and a reduction in βEP neurons in alcohol treated rats, in comparison to the control or non-treated animal models. However, the exosomes displayed an inability to colocalize within the βEP neurons in both alcohol and non-treated animals. Therefore, we found that genetically modified exosomes can bypass the BBB and travel into the brain to localize into the hypothalamic arcuate area, more so through alcohol administration, and affect glial and βEP communication. Further research in observing where the exosomes are colocalizing is essential in finding what other roles the exosomes are involved in.

DEDICATION

I dedicate this work to my mother, Brigida Nuñez, for her constant support throughout my graduate career, and nonstop perseverance in providing me the best life she could. I also want to acknowledge my step-father, Angel Martinez, for coming into our family when I was very young and for becoming the father figure I did not have growing up.

I dedicate this work to my two sisters, Chany Denisse and Ashley Martinez, who were my constant source of communication and always found a way to make me smile through times of stress.

I dedicate this work to my uncle, Angel Nuñez, for always checking in on me and making sure I was okay. Also, for taking me on mini-vacations with his family throughout the year for fun times.

I dedicate this work to a woman who I consider to be my second mother, Mirian Crespo. She has been a part of my family since I was born and is my motivation for continuing on in my studies to become a doctor. I hope one day I can heal her from her ongoing dementia.

Lastly, I dedicate this work to my late grandmother, Anna Brito, who passed away last Fall semester on October 4, 2019. She was the backbone to my family. I learned a great deal from her and loved all of our conversations. She was always happy and full of energy, and constantly reminded me how proud she was of all my achievements. I miss her very much. Rest in peace, Mama.

ACKNOWLEDGEMENT

I would like to acknowledge Dr. Sarkar for allowing me to enter his lab and learn from him and his post-docs. He was a great advisor and teacher. I am grateful I had the privilege of being one of his students given his distinguished role not just at Rutgers, but across the nation.

I want to acknowledge postdoc Sayani Mukherjee from the Sarkar lab. She taught me the protocols necessary to carry out the experiment and helped me with learning everything I needed to know about this subject. She was always available, and I am pleased to have had her assistance throughout the year.

I want to acknowledge everyone in the Sarkar lab who helped me along the way, whether it was in teaching me a protocol or answering my questions. They were always very welcome and willing to assist students with their research.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

AD- AD-LIBITUM
AD-EX- AD LIBITUM WITH EXOSOMES
AF- ALCOHOL-FED
AF-EX- ALCOHOL FED WITH EXOSOMES
AJ- ADHERENS JUNCTION
ANS- AUTONOMIC NERVOUS SYSTEM
ARBD- ALCOHOL-RELATED BIRTH DEFECTS
ARND- ALCOHOL-RELATED NEURODEVELOPMENTAL DISORDER
ATP- ADENOSINE TRIPHOSPHATE
BBB- BLOOD-BRAIN BARRIER
βEP- BETA-ENDORPHINS
CNS- CENTRAL NERVOUS SYSTEM
CSF1R- COLONY STIMULATING FACTOR 1 RECEPTOR
DAMP- TISSUE-DAMAGE ASSOCIATED MOLECULAR PATTERN
Dox- DOXORUBICIN
ERK- EXTRACELLULAR RECEPTOR SIGNALING KINASES
ESCRT- ENDOSOMAL SORTING COMPLEX REQUIRED FOR TRANSPORT
EV- EXTRACELLULAR VESICLE
FAS- FETAL ALCOHOL SYNDROME
FASD- FETAL ALCOHOL SPECTRUM DISORDER
HSP-70- HEAT-SHOCK PROTEIN 70
iMDC- IMMATURE DENDRITIC CELL
JAM- JUNCTION ADHESION MOLECULE
LAMP2B- LYSOSOME-ASSOCIATED MEMBRANE GLYCOPROTEIN 2B
MBH- MEDIOBASAL HYPOTHALAMIC
MHC- MAJOR HISTOCOMPATIBILITY COMPLEX
MVB- MULTIVESICULAR BODY
ND-PAE- NEUROBEHAVIORAL DISORDER ASSOCIATED WITH PRENATAL ALCOHOL EXPOSURE
PAMP- PATHOGEN ASSOCIATED MOLECULAR PATTERN
PNS- PERIPHERAL NERVOUS SYSTEM
POMC- PROOPIOMELANOCORTIN
PRR- PATTERN RECOGNITION RECEPTOR
TJ- TIGHT JUNCTION

INTRODUCTION

CHAPTER 1. BACKGROUND

1.1 FETAL ALCOHOL SPECTRUM DISORDERS DEFINITION AND EPIDEMIOLOGY

Fetal Alcohol Spectrum Disorders (FASDs) covers a spectrum of disorders that affect children due to their mothers' consuming alcohol throughout pregnancy (Wilhoit et al., 2017). These disorders can be categorized into four groups based on symptoms: Fetal Alcohol Syndrome (FAS), Alcohol-Related Neurodevelopmental Disorder (ARND), Alcohol-Related Birth Defects (ARBD), and Neurobehavioral Disorder Associated with Prenatal Alcohol Exposure (ND-PAE). These disorders are mainly characterized by behavioral and learning impairments that develop as the child matures in age, in addition to several physical changes, such as low body weight, short height, and a smaller head size (Murawski et al., 2015). Children with FASDs struggle to maintain healthy social relationships and often have poor memory and reasoning skills, as well as, delayed speech and language (CDC 2019). These characteristics are a result of structural changes in the brain due to alcohol exposure, which may include damage to the basal ganglia and cerebellum. Children with ARND or ARBD may struggle intellectually and have problems learning, or develop organ-specific problems (ears, kidneys, bones), respectively. Children with ND-PAE have problems in thinking, behavior, mood changes, and daily activities, such as getting dressed or bathing (CDC 2019). In more severe cases, such as children with FAS, there can be permanent brain damage accompanied by these

characteristics, where the children struggle with difficulties in all aspects physically, mentally, socially, and healthwise (Wilhoit et al., 2017).

According to the CDC (2019), about 40,000 children are born with an FASD in the United States every year, with 1 in 9 pregnant women binge-drinking alcohol (4-5 drinks). There are high estimates in certain areas of the United States, with FAS affecting 6-9 out of 1000 children (CDC 2019). The cost to live with FAS, one disorder out of the FASDs, was over $2 million dollars per individual and $4 billion dollars annually in the United States (Lupton 2004). This emphasizes the importance of focusing on preventing or reducing the occurrence of FASDs.

1.2 HYPOTHALAMUS AND BETA-ENDORPHINS

1.2.1 HYPOTHALAMUS, ARCUATE NUCLEUS, AND POMC

The hypothalamus is an important brain structure involved in the autonomic nervous system (ANS) and functions to control homeostasis and behavior (Xie et al., 2017). It plays an integral role in the endocrine system by controlling hormone release from the pituitary gland. Each region of the hypothalamus has specific neurons and nuclei with particular functions (Burbridge et al., 2016). One area of interest is the tuberal hypothalamus, which contains the arcuate nucleus, and is involved in energy balance and stress (Xie et al., 2017). The arcuate area consists of many types of neurons, including the melanocortin neurons, which play an important role in regulating metabolism (Xie et al., 2017). The proopiomelanocortin (POMC)-expressing neurons have a distinct function in producing opioid peptides like βEPs (Pastor et al., 2014). POMC is a pro-hormone that

functions in reducing food intake and increasing the use of energy (Toda et al., 2017). In this review, we focused on how βEP function is affected before and after alcohol exposure.

1.2.2 BETA-ENDORPHINS BACKGROUND

βEPs are neuropeptides in the anterior pituitary gland involved in pain management and reward behaviors like feeding and drinking. In the peripheral nervous system (PNS), βEPs can produce analgesia, or the inability to feel pain, by binding to opioid receptors, mu and delta, on pre- and post-synaptic nerve terminals (Sprouse-Blum et al., 2010). This leads to the inhibition of tachykinins from being released, such as substance P, a protein involved in pain transmission (Stein 1995). In the central nervous system (CNS), βEPs mainly bind to the mu-opioid receptors and exert their analgesic effect at the presynaptic nerve terminal. However, instead of releasing substance P, the βEPs inhibit the release of GABA, an inhibitory neurotransmitter, resulting in an excess of dopamine production (Sprouse-Blum et al., 2010). Furthermore, studies have shown that immune cells are capable of producing βEPs during immune responses like inflammation (Shaaban et al., 2004).

1.2.3 EFFECT OF ALCOHOL ON BETA-ENDORPHINS

Endogenous opioid peptides, including βEPs, are known to function within the mesolimbic reward system, which is important during alcohol consumption (Zalewska-Kaszubska et al., 2005). In a study done by Mendez et al., they discovered that a single dose of ethanol administered to rat animal models increased βEP production and opioid peptide gene expression in the brain (Mendez et al., 2001). On the other hand, prolonged alcohol consumption has the opposing effects on βEPs through causing apoptosis of βEP

neurons (Aguirre et al., 1995, Sarkar et al., 2007). It is suggested that microglia might play a role in this βEP neurodegeneration due to how βEPs also play an important role in responding to stress (Sarkar et al., 2014). Low levels of βEP could lead to more alcohol consumption or anxiety during alcohol withdrawal (Grisela et al., 1999, Kiefer et al., 2002, Zalewska-Kaszubska et al., 2005). Additionally, the βEP precursor, POMC, increases with alcohol consumption, but decreases with long-term alcohol exposure (Ekman et al., 1994, Winkler et al., 1995). This leads to βEP degradation and dysregulatory effects of their overall function (Winkler et al., 1995).

1.3. MICROGLIA

1.3.1 MICROGLIA FUNCTION

Microglia comprise the majority of brain immune cells and play an essential role in innate immunity (Vetreno et al., 2013). Microglia are able to communicate with neurons in the CNS to detect environmental changes, remove dead cells and debris through phagocytosis, and regulate angiogenesis (Colonna et al., 2017, Eyo et al., 2013). Microglia depend on the colony stimulating factor 1 receptor (CSF1R) which activates intracellular signals and kinases, such as extracellular signal-regulated kinases (ERK), in order to promote microglial maintenance and development (Colonna et al., 2017). Unless activated by the neurons in the CNS, microglia tend to remain in a quiescent state where they monitor their surrounding cells. Once activated by an injury or inflammation, the microglial cell bodies enlarge into an amoeboid shape in order to cover more area (Colonna et al., 2017). The phenotype of microglia during activation is also important in knowing what particular role the microglia will carry out (Raivich et al., 1999). Raivich

et al. have identified the five stages of microglial activation as: resting (stage 0), alert (stage 1), homing (stage 2), phagocytic (stage 3a) and bystander activation (stage 3b). The microglia can transform from a stout and unramified form into full phagocytes depending on the injury or progression of infection (Raivich et al., 1999). For the highest level of activation, proinflammatory factors such as IL-1Beta, TNF-alpha, and prostaglandins are expressed in the microglia, whereas, in lower levels of activation, neuroprotective factors like IL-10, TNF-beta, and neurotrophins are released (Block et al., 2005).

1.3.2 COMMUNICATION BETWEEN MICROGLIA AND NEURONS

Many reports have shown that neurons modulate the activation of microglia and induce microglial functions by secreting signaling factors, neurotransmitters, and purines (Eyo et al., 2013). Neurons are able to keep the microglia in its resting state until an event occurs where microglia need activation. Moreover, excitatory and inhibitory neurotransmission can alter the motility and activity of microglia, which is suggested to be through the use of adenosine triphosphate (ATP) (Fontainhas et al., 2011). Microglia express a wide range of immune receptors that detect changes in the environment and induce specific responses in the CNS. Some receptors are pattern-recognition receptors (PRRs) that detect pathogen-associated or tissue damage-associated molecular patterns (PAMPs and DAMPs, respectively), and chemokine receptors that control microglia positioning and enhance their ability to bind to target cells (Colonna et al., 2017).

1.3.3 ALCOHOL-INDUCED ACTIVATION OF MICROGLIA

Prolonged alcohol consumption can cause neuroinflammation which induces activation of microglia. In one study done by Marshall et al., they found that although the microglia did not fully activate into macrophages when the rats were exposed to alcohol
for four days, there was an increase in microglial proliferation following this time period (Marshall et al., 2013). Their experiment highlighted that alcohol-induced microglial activation occurs as a result of alcohol-induced cell death (Marshall et al., 2013). To further confirm this statement, a study done by Sarkar et al. 2010, found that when a rat cell culture of mediobasal hypothalamic (MBH) cells were treated with alcohol, microglia were able to fully activate and induce apoptosis on the MBH neurons (Sarkar et al., 2010).

Heavy alcohol can also cause oxidative stress in developing neurons by generating microglial reactive oxidative species (Sarkar et al, 2014). Oxidative stress is defined as the imbalance between free radicals and antioxidants. Free radicals are molecules with an uneven number of electrons enabling them to have increased chemical reactivity with another molecule (Betteridge 2000). Antioxidants are molecules that inhibit the oxidation of a molecule, in this case the free radical, by donating an electron to reduce reactivity (Betteridge 2000). Alcohol exposure on developing hypothalamic neurons increases cell apoptosis via oxidative stress and increased microglial production (Sarkar et al., 2014).

1.4 EXOSOMES

1.4.1 EXOSOME BACKGROUND AND FUNCTION

Extracellular Vesicles (EVs) are membrane-bound nanovesicles that originate from various cellular components, such as endosomes or the cellular membrane (Pleet et al., 2018). The three main types of EVs are microvesicles, apoptotic bodies, and exosomes. Each of these vary in size, development, and function. Microvesicles release through budding of the cell membrane; apoptotic bodies release from dying cells; and exosomes release from late endosomes, creating multivesicular bodies (MVBs) (Pleet et al., 2018). Endosomes are a population of endocytic vacuoles through which molecules travel to lysosomes for degradation (Helenius et al., 1983). In this review we focused primarily on exosomes.

Exosomes range from 30 to 150 nm in size, and are formed through inward, or reverse, budding of endosomal membranes to generate MVBs (Shenoda et al., 2016). They have a similar lipid bilayer as that of a cell membrane and are rich in lipids, like gangliosides and sphingomyelin, and cholesterol (Zaborowski et al., 2015). Due to their lipid bilayer composition, exosomes are able to carry transmembrane proteins and receptors to communicate with other cells, as well as, contain different types of RNA (Pitt et al., 2016, Valadi et al., 2007). Some proteins include tetraspanins, major histocompatibility complex (MHC) class II proteins, and accessory proteins TSG101 and Alix, which are associated with the endosomal sorting complex required for transport (ESCRT) pathway (Abels et al., 2016).

The internal content and biological effect of an exosome depends on the source or type of cell that it was derived from (Shenoda et al., 2016). Exosomes have several ways of communicating with their target cells, including receptor-ligand interactions, attachment to the cell, or endocytosis. Through a receptor-ligand interaction the exosome

is able to present a protein receptor on its surface to target the recipient cell. Exosomes may also fuse with their target cell to deliver their surface proteins, or cytoplasm in some cases. Lastly, exosomes are able to be phagocytosed by their target cell to induce a specific function (Valadi et al., 2007). Their main functions are to discard unnecessary cell components during cell maturation, mediate cell-to-cell communication, and in neurons, modulate synaptic plasticity (Frühbeis et al., 2012, Li et al., 2006).

1.4.2 ROLE IN CNS AND IMMUNE RESPONSE

The immune system is categorized into two distinct types of responses: innate and adaptive. The innate response is faster and attacks pathogens nonspecifically, while the adaptive response is slower, pathogen-specific, and can develop immunological memory (Netea et. al., 2016). There are many cell types in the neuroimmune system that can secrete exosomes and stimulate or suppress the immune system (Li et al., 2006). Neurotransmitter release stimulates oligodendrocytes to release lipid-derived exosomes capable of carrying enzymes that contribute to neuroprotection. Astrocytes release exosomes carrying proteins associated with their synaptic maintenance function, such as synapsin1 and heat-shock protein 70 (HSP70). Microglia release exosomes containing proteins that respond to immunological triggers (Frühbeis et al., 2012). Through microglial activation, exosomes play a role in pathogenesis, neurodegeneration, and induce inflammatory reactions in the brain (Brites et al., 2015). Nonetheless, exosome composition is subject to change in the presence of an infection, inflammation, or tumor (Shenoda et al., 2016).

1.4.3 MODIFICATION OF EXOSOMES

As a result of exosomes providing a broad range of functions throughout the body, they can be genetically modified to communicate with distinct cells or induce specific reactions. Exosomes have demonstrated a high efficiency in being taken up by recipient calles and used as vehicles to transport therapeutic agents or molecules. the endosomal pathway in which exosomes are released can be modified to enable them to carry a gene, protein, or drug of interest (Koppers et al., 2013). Recent studies have indicated viral and non-viral techniques used to modify the parent cell secreting the exosome to influence the exosome content, along with modifying the exosome surface markers to target tumor cells (Gilligan et al., 2017).

The most common non-viral methods to modify exosome content include incubation, electroporation, and lipofection. During incubation, either the parent cell is incubated with the drug being studied or the exosomes are isolated then incubated with the drug. In this method the drug must be small enough to bypass the membrane of the exosome (Gilligan et al., 2017). In electroporation, a drug is incubated with the exosomes and an electrical field is applied, thus creating pores for the drug to enter (Lamichhane et al., 2015). In lipofection, liposomes carry the content designed for transfection into the exosomes (Felgner et al., 1987). The two types of viral methods include retroviral and adenoviral vectors. Retroviral vectors are efficient in promoting transduction and maintaining transgene expression. Adenoviral vectors are capable of transducing dividing and non-dividing cells (Gilligan et al., 2017).

Surface modification of exosomes involves engineering their parent cell to express certain markers to support exosomal delivery to target cells.

For instance, in a study done by Tian et. al, exosomes derived from mouse immature dendritic cells (imDCs) were modified to express an exosome membrane protein, lysosome-associated membrane glycoprotein 2b (Lamp2b), fused with a specific αv integrin peptide, iRGD (Tian et al., 2014). Isolated exosomes were loaded with the chemotherapeutic drug, Doxorubicin (Dox) through electroporation. Through intravenous injection, the modified exosomes were able to successfully deliver Dox to the targeted tumors, resulting in reduced tumor growth (Tian et al., 2014).

1.4.4 ALCOHOL AND EXOSOMES

Alcohol consumption stimulates microglia to release exosomes as a stress response. In a study done by Crenshaw et. al, they found that alcohol affects microglial cell content and morphology, and negatively affects exosome composition and biogenesis (Crenshaw et al., 2019). Although their microglial cells were undergoing apoptosis, exosome composition did not change (Crenshaw et al., 2019). This highlights the importance of understanding the relationship between alcohol and microglia, and alcohol-induced exosome release. In addition, Momen-Heravi et al. found that exosome deposition increased after a binge alcohol period in hepatocytes, along with miRNA expression (Momen-Heravi et al., 2015). In our study, we focused on alcohol-induced exosome deposition in the hypothalamic area of the brain.

1.5 BLOOD-BRAIN BARRIER

1.5.1 BBB FUNCTION

The Blood-Brain Barrier (BBB) functions to maintain a homeostatic environment for the brain (Andreone et al., 2017). The BBB also regulates transportation of molecules

from blood circulation to the brain and clears waste from the brain into circulation (Zhao et al., 2017). It is composed of epithelial cells held together by tight junctions and adherens junctions that control molecular transport. Tight junctions (TJs) are composed of transmembrane proteins including junction adhesion molecules (JAMs), claudins, and occludins. TJs prevent paracellular passage of hydrophilic molecules across the BBB and ensure structural integrity (Singh et al., 2007, Andreone et al., 2017). Adherens junctions (AJs) modulate cell-to-cell adhesion through many types of integral membrane proteins, specifically cadherins and nectins. AJs promote endothelial and vascular integrity through control of cell morphogenesis (Ivanov et al., 2013). In the BBB there is a balance between paracellular and transcellular passage of molecules. In pathological conditions, BBB integrity could be compromised causing processes such as neuroinflammation, neurodegeneration, and ion imbalance (Daneman et al., 2015). The restrictive nature of the BBB makes it difficult for drugs to enter the CNS, therefore, many efforts have been made to bypass the BBB for therapeutic delivery (Daneman et al., 2015).

1.5.2 BBB AFTER ALCOHOL EXPOSURE

Alcohol consumption over a prolonged period negatively affects the integrity of the BBB (Gulati et al., 1985). Infections and brain damage are more likely to occur if this protective barrier is compromised. Many *in vitro* studies have indicated that short and long-term ethanol exposure results in phosphorylation of TJ proteins, thus altering the TJ architecture and increasing the permeability of the BBB (Singh et al., 2007, Haorah et al., 2005). Excessive alcoholism can ultimately lead to neurobehavioral disorders, neuroinflammation and oxidative stress (which also promotes TJ phosphorylation). (Singh et al., 2007, Haorah et al., 2005). Likewise, the higher the concentration of the

alcohol and the longer the length of its alkyl chain, the more probability the alcohol has to increase BBB permeability and more damaging the effects on neural function (Gulati et al., 1985).

1.5.3 EXOSOME INVOLVEMENT IN BBB

Exosomes are noted for being able to promote cell-to-cell communication across the body (Pitt et al., 2016). However, the role of exosomes in traveling across the BBB was poorly understood until more recently. Zhao et al. found that neuronal exosomes, derived from zebrafish, were able to travel across endothelial cells in the BBB and transfer material to other neurons (Zhao et al., 2017). They also discovered that an adherens junction molecule, vascular endothelial cadherin, was downregulated, which they believe may have possibly played a role in helping the exosomes cross the BBB (Zhao et al., 2017). Brain endothelial cell-derived exosomes have also been evaluated for cancer treatments. Exosomes have been effective in overcoming the BBB to deliver drugs or therapeutic agents to cells in the brain (Yang et al., 2015). This depicts the ability of exosomes to be used as mediators for medical treatment.

HYPOTHESIS

Exosomes derived from genetically modified microglial cells can be introduced through the periphery to cross the BBB and influence BEP and glial interaction in the brain, and alcohol administration increases the deposition of exosomes across in the hypothalamic area.

AIMS OF THIS STUDY

Aim 1: To determine if exosomes derived from genetically modified microglial cells can be introduced into the blood circulation and cross the BBB, and verify that alcohol administration increases the deposition of exosomes into the brain

Aim 2: To establish the role of exosomes in glial and βEP communication

CHAPTER 2

2.1 MATERIALS AND METHODS

Animals. Adult Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). All animals were kept under standard lighting conditions (12-hour lights on; 12-hour lights off) and provided rodent chow and water ad libitum. These rats were bred to generate neonatal animals, which were used in this study. Animal care and treatment were performed in accordance with institutional guidelines, and protocols were approved by the Rutgers Institutional Animal Care and Facilities Committee and complied with National Institutes of Health policy.

In vivo studies. Postnatal rat pups (PND2; both sexes) were fed by gavage a milk formula containing 11.34% ethanol (vol/vol; 0.1-0.2 ml/animal; during a period of 1 minute), yielding a total daily ethanol dose of 2.5 g/kg (AF), or they were left in the litter with their mother (AD) as described by us previously (Chastain et al., 2019; Shrivastava et al., 2017). (Alcohol-fed with exosomes (AF-EX), Control with exosomes (AD-EX)). Gavage feeding was conducted at 10:00 AM and 12:00 PM from PND 2-6. After 2 hours of the last gavage, rats were injected with 50ul of GFP-labeled exosomes intra-cardially (GFP-labeled exosomes provided by Dr. Ilker Sariyer from Temple University). After a period of 30-45 minutes, the pups were sacrificed at the mediobasal hypothalamus (MBH; the mediobasal portion of the hypothalamus extended approximately 1 mm rostral to the optic chiasma and just caudal to the mammillary bodies, lateral to the hypothalamic sulci, and dorsal to 2 mm deep) was collected for immune-histochemical studies.

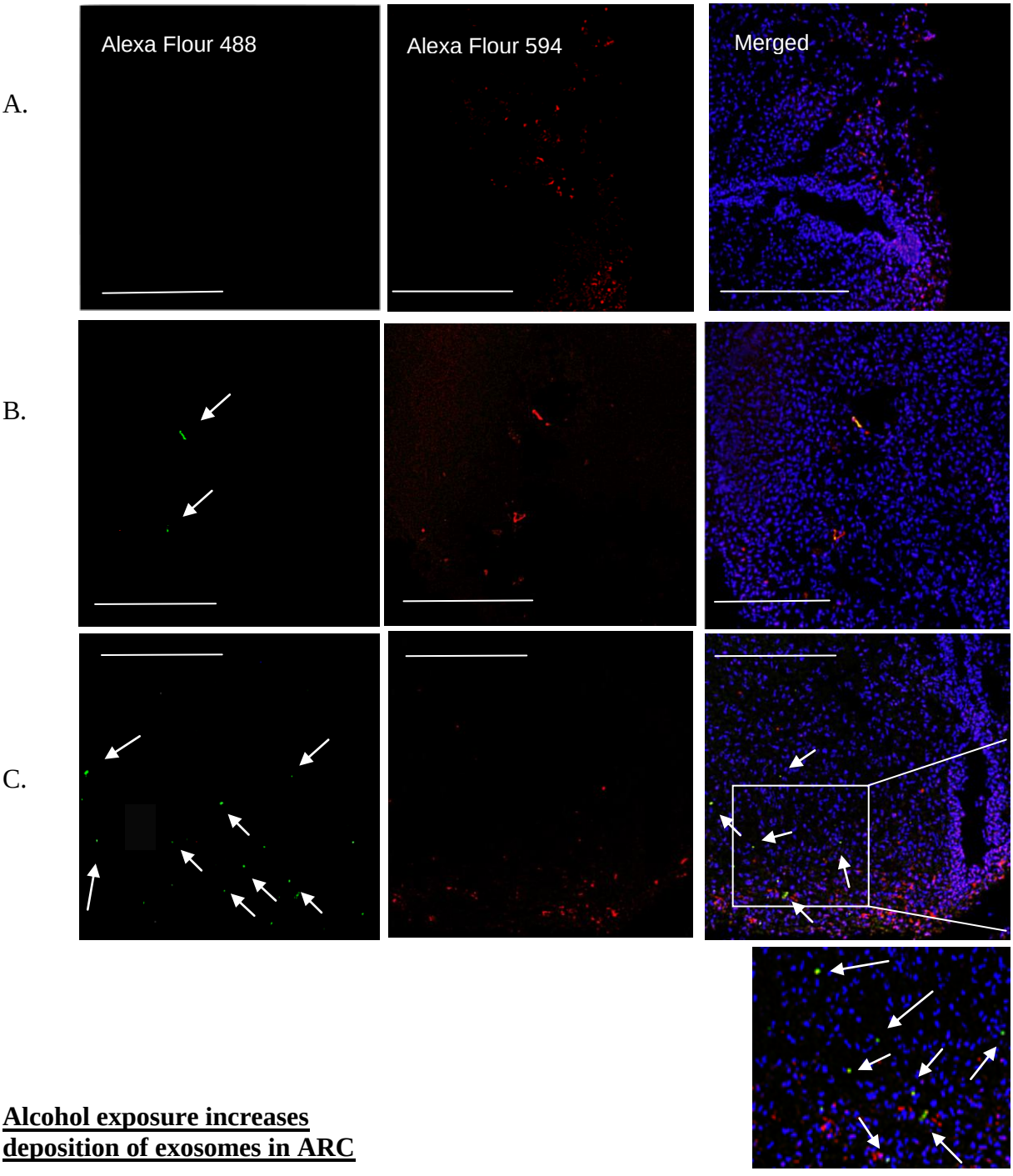
Immunohistochemistry for β-endorphin. Serial coronal sections of non-perfused brains were made using a Leica cryostat at 30 µm in thickness from stereotaxic plates 19 to plates 23 (Bregma −2.3 to −4.3 mm) spanning the arcuate nucleus. Perfused sections were mounted on Superfrost Plus glass slides (VWR, Radnor, PA) containing one AD, and one AF brain section. The sections were washed in PBS twice followed by antigen retrieval in a citrate buffer (pH 6.2) at 100°C for 20 minutes. After two washes in PBS-T (0.05% Triton-X), the sections were incubated in a blocking buffer (2.5% normal horse serum in PBS-T) at room temperature for one hour. The sections were subsequently incubated overnight at 4 °C with the rabbit anti-β-endorphin and mouse-anti-GFP (1:1000 each; Peninsula Laboratories, San Carlos, CA). After the primary antibody incubation, samples were washed in PBST and then sections were incubated with an Alexa Fluor 594 donkey anti-rabbit and Alexa Fluor 488 donkey anti-mouse secondary antibody (1:500; Thermo Fisher Scientific, Grand Island, NY) for 1 hr. Sections were mounted with DAPI (Vector Laboratories, Burlingame, CA) and sealed with nail polish. To evaluate the immunohistochemical staining intensity, animals in each experimental group were photographed using Nikon- TE 2000 inverted microscope (Nikon Instruments Inc., Melville, NY). Cell counting was quantified using ImageJ software (National Institutes of Health, Bethesda, MD).

Statistical analysis. Results are expressed as Mean ± SEM. One-way ANOVA with Newman Keuls post hoc analysis was used to analyze the differences between multiple groups. The value $P < 0.05$ and onward was considered significant. Data were analyzed using Prism 5.0 (Graph Pad Software).

2.2 RESULTS

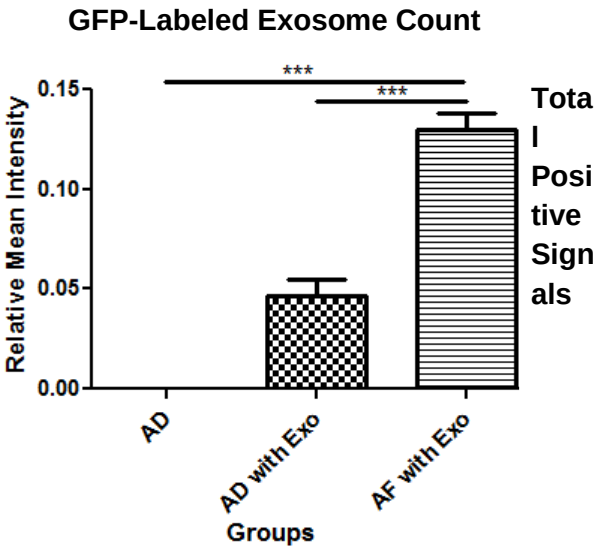
2.2.1 AIM 1 RESULTS

Using postnatal ethanol exposure rat pups (PND2-human third trimester equivalent), I determined the number of exosomes deposited into the brain in control (AD, AD with Exo) and alcohol-fed (AF with Exo) animals. Exosomes can travel across the BBB for neuronal communication, and more so with alcohol administration (Zhao et al., 2017). The AF group displayed a higher count of GFP-labeled exosomes in the hypothalamic arcuate nucleus area, versus the AD with Exo group (Fig. 1 B, C and 2). The AD group did not have any exosomes injected (Fig. 1A). Therefore, exosomes, derived from genetically modified microglial cells, were capable of successfully traveling across the BBB, and postnatal ethanol exposure increases BBB permeability by enabling more exosomes to enter the brain.



Alcohol exposure increases deposition of exosomes in ARC

Figure 1. A.) AD (without exosomes), B.) AD with Exosomes, C.) AF with Exosomes Immunofluorescence staining showing alcohol exposure increased the deposition of GFP labeled exosomes in the arcuate nucleus of the AF groups whereas, AD groups showed a little or no change. βEP neurons are visible in red (Alexa Flour 594), exosomes in green (Alexa Flour 488), and nucleus in blue (DAPI). Arrows are showing GFP labeled exosomes in arcuate nucleus. Size depicted is 100um.
The images are taken in 10X magnification by fluorescent microscope.



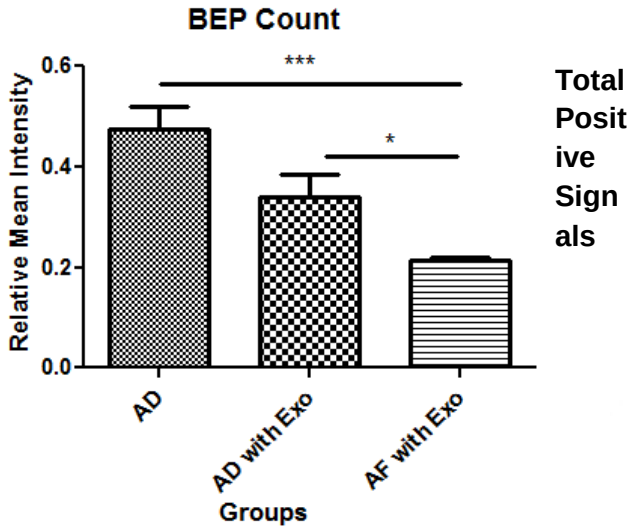
Alcohol exposure increases deposition of GFP-labeled exosomes in ARC

Figure 2. Bar diagram showing increased deposition of GFP labeled exosomes in alcohol treated groups, versus control groups.
N=3 *, P<0,05; ***P<0.001

2.2.2 AIM 2 RESULTS

Using the AD, AD with Exo, and AF with Exo groups, I determined the role of exosomes in glial and βEP communication by evaluating the number of βEPs in each group, and whether the exosomes were able to colocalize into the βEPs. In the CNS, microglia release exosomes during alcohol exposure as a stress response, and they can also induce βEP neurodegeneration and apoptosis (Crenshaw et al., 2019, Sarkar et al., 2014). In comparison to the AD group, the AF with Exo group showed a major depletion in the number of βEP present in the hypothalamic arcuate nucleus area (Fig 1.A, C and Fig 3). The AD with Exo group had a slight decrease in βEP neurons, but not as much as the AF with Exo group (Fig. 1B and 3). The exosomes did not manage to colocalize within the βEP neurons (Pearson's Coefficient = 0, data not shown). Even though the exosomes traveled into the hypothalamic arcuate area, another factor must be introduced to ensure proper neuronal communication with βEPs.

Consequently, βEPs are negatively affected when exposed to alcohol, which leads to the assumption that βEP neurons might have undergone cell death.



Alcohol exposure decreases BEP count in ARC

Figure 3. Statistical analysis show decreased BEP count in alcohol treated group in comparison to control group.
N=3*, P<0,05; ***,P<0.001

DISCUSSION AND CONCLUSIONS

Exosomes are important in assisting with cell-to-cell communication and neuroimmune responses. In the immune system, exosomes can be secreted by various types of cells to induce or suppress a specific reaction (Li et al., 2006). In the CNS, the BBB provides a barrier to prevent infection and certain molecules from entering the brain. If the BBB integrity is compromised, blood-borne toxins may pass through and negatively affect brain function (Andreone et al., 2017). Factors that promote this include, neuroinflammation, oxidative stress, and diseases like Alzheimer's. Prolonged alcohol exposure has been shown to increase BBB permeability (Gulati et al., 1985).

Given that almost every cell in the body can produce exosomes, genetic modification is essential to understand what other functions exosomes can carry out, as well as, what cells they can communicate with. Through using exosomes, derived from genetically modified microglial cells, and injecting them intracardially into postnatal rat models, I found that the exosomes were able to travel through the BBB efficiently. However, when alcohol was administered to the animals, the number of exosomes that deposited into the brain increased significantly in alcohol treated groups versus control groups. This showed that the exosomes were capable of successfully traveling across the BBB, and that alcohol exposure increases their deposition due to compromised BBB integrity.

Microglia are the major macrophages of the neuroimmune system. These cells coordinate immune responses, regulate angiogenesis, and remove debris from the CNS (Colonna et al., 2017). Microglia can secrete exosomes during stress and induce

neuroinflammation and neurodegeneration of cells (Brites et al., 2015). The role of microglia with βEPs is not quite clear, but during prolonged alcohol exposure, microglia can induce βEP neuron apoptosis (Aguirre et al., 1995). The alcohol treated groups in this experiment had a significant decrease in βEP levels, when compared to the control groups. This suggests that the βEP neurons are undergoing apoptosis or neurodegeneration.

Lastly, although the exosomes were able to travel into the brain, they were not able to localize within the βEP cells of all the groups. This could likely be due to the exosomes lacking a neuronal factor to communicate with the correct neurons in the hypothalamic arcuate nucleus area. Further research must be done to understand why the exosomes did not localize into the βEP neurons, and where they might have localized instead.

These experiments demonstrated that the use of exosomes is an important topic to examine when it comes to scientists discovering methods to surpass the BBB and deliver molecules or therapeutic agents to neurons in the CNS. Prolonged alcohol exposure causes damaging effects in the brain, especially during pregnancy, and can lead to the child developing behavioral and learning disorders as they develop (Murawski et al., 2015). More cases of FAS are being diagnosed every year, and there is still no cure, but there is more research to be done to find one.

REFERENCES

Abels, Erik R, and Xandra O Breakefield. “Introduction to Extracellular Vesicles: Biogenesis, RNA Cargo Selection, Content, Release, and Uptake.” *Cellular and molecular neurobiology* vol. 36,3 (2016): 301-12. doi:10.1007/s10571-016-0366-z

Aguirre, J. C., et al. “Classification of Alcoholics on the Basis of Plasma Beta-Endorphin Concentration.” *Elsevier Science, Inc.*, vol. 12, no. 6, 20 Apr. 1995, pp. 531–534., doi:0741-8329(95)00039-9 .

A. M. Fontainhas, M. Wang, K. J. Liang et al., “Microglial morphology and dynamic behavior is regulated by ionotropic glutamatergic and GABAergic neurotransmission,” PLoS ONE, vol. 6, no. 1, Article ID e15973, 2011.

Andreone BJ, Chow BW, Tata A, et al. Blood-Brain Barrier Permeability Is Regulated by Lipid Transport-Dependent Suppression of Caveolae-Mediated Transcytosis. *Neuron*. 2017;94(3):581–594.e5. doi:10.1016/j.neuron.2017.03.043

Ashok K Singh, Yin Jiang, Shveta Gupta, Elhabib Benlhabib, Effects of chronic ethanol drinking on the blood–brain barrier and ensuing neuronal toxicity in alcohol-preferring rats subjected to intraperitoneal LPS injection, *Alcohol and Alcoholism*, Volume 42, Issue 5, September 2007, Pages 385–399, https://doi.org/10.1093/alcalc/agl120

Betteridge, D. John. “What Is Oxidative Stress?” *Metabolism*, vol. 49, no. 2, Feb. 2000, pp. 3–8., doi:10.1016/s0026-0495(00)80077-3.

Brites, Dora, and Adelaide Fernandes. “Neuroinflammation and Depression: Microglia Activation, Extracellular Microvesicles and microRNA Dysregulation.” *Frontiers in cellular neuroscience* vol. 9 476. 17 Dec. 2015, doi:10.3389/fncel.2015.00476

Burbridge S., Stewart I. and Placzek M. (2016). Development of the neuroendocrine hypothalamus. Compr. Physiol. 6, 623-643. 10.1002/cphy.c150023

Center for Disease Control and Prevention (2019). *Fetal alcohol spectrum disorders*. Retrieved from http://www.cdc.gov/ncbddd/fasd/index.html.

Chastain, Lucy G., and Dipak K. Sarkar. “Role of Microglia in Regulation of Ethanol Neurotoxic Action.” *International Review of Neurobiology Neuroimmune Signaling in Drug Actions and Addictions*, vol. 118, 2014, pp. 81–103., doi:10.1016/b978-0-12-801284-0.00004-x.

Colonna M, Butovsky O. Microglia Function in the Central Nervous System During Health and Neurodegeneration. Annu Rev Immunol. 2017;35:441–468. doi:10.1146/annurev-immunol-051116-052358

Daneman, Richard, and Alexandre Prat. “The blood-brain barrier.” *Cold Spring Harbor perspectives in biology* vol. 7,1 a020412. 5 Jan. 2015, doi:10.1101/cshperspect.a020412

Crenshaw, Brennetta J et al. “Alcohol Modulates the Biogenesis and Composition of Microglia-Derived Exosomes.” *Biology* vol. 8,2 25. 27 Apr. 2019, doi:10.3390/biology8020025

Dipak K. Sarkar, Peter Kuhn, Jasson Marano, Cuiping Chen, Nadka Boyadjieva, Alcohol Exposure during the Developmental Period Induces β-Endorphin Neuronal Death and Causes Alteration in the Opioid Control of Stress Axis Function, *Endocrinology*, Volume 148, Issue 6, 1 June 2007, Pages 2828–2834, https://doi.org/10.1210/en.2006-1606

Ekman, Ann-Charlotte, et al. “Delayed Pro-Opiomelanocortin Activation after Ethanol Intake in Man.” *Alcoholism: Clinical and Experimental Research*, vol. 18, no. 5, Oct. 1994, pp. 1226–1229., doi:10.1111/j.1530-0277.1994.tb00109.x.

Eyo UB, Wu LJ. Bidirectional microglia-neuron communication in the healthy brain. Neural Plast. 2013;2013:456857. doi:10.1155/2013/456857

Felgner, P L et al. “Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure.” *Proceedings of the National Academy of Sciences of the United States of America* vol. 84,21 (1987): 7413-7. doi:10.1073/pnas.84.21.7413

Frühbeis C, Fröhlich D, Krämer-Albers EM. Emerging roles of exosomes in neuron-glia communication. *Front Physiol*. 2012;3:119. Published 2012 Apr 30. doi:10.3389/fphys.2012.00119

Gilligan, Katie E, and Róisín M Dwyer. “Engineering Exosomes for Cancer Therapy.” *International journal of molecular sciences* vol. 18,6 1122. 24 May. 2017, doi:10.3390/ijms18061122
Helenius, Ari, et al. “Endosomes.” *Trends in Biochemical Science*, vol. 8, no. 7, July 1983, pp. 245–250., doi:10.1016/0968-0004(83)90350-X.

Grisel, Judith E., et al. “Ethanol Oral Self-Administration Is Increased in Mutant Mice with Decreased β-Endorphin expression1Published on the World Wide Web on 1 April 1999.1.” *Brain Research*, vol. 835, no. 1, 17 July 1999, pp. 62–67., doi:10.1016/s0006-8993(99)01384-0.

Gulati, Anil, et al. “Effect of Alcohols on the Permeability of Blood-Brain Barrier.” *Pharmacological Research Communications*, vol. 17, no. 1, Jan. 1985, pp. 85–93., doi:10.1016/0031-6989(85)90054-2.

Haorah, J. “Alcohol-Induced Oxidative Stress in Brain Endothelial Cells Causes Blood-Brain Barrier Dysfunction.” *Journal of Leukocyte Biology*, vol. 78, no. 6, 4 Oct. 2005, pp. 1223–1232., doi:10.1189/jlb.0605340.

Ivanov AI, Naydenov NG. Dynamics and regulation of epithelial adherens junctions: recent discoveries and controversies. *Int Rev Cell Mol Biol*. 2013;303:27–99. doi:10.1016/B978-0-12-407697-6.00002-7

Jadwiga, and Elżbieta Czarnecka. “Deficit in Beta-Endorphin Peptide and Tendency to Alcohol Abuse.” *Peptides*, vol. 26, no. 4, Apr. 2005, pp. 701–705., doi:10.1016/j.peptides.2004.11.010.

Kable, J. A., O’Connor, M. J., Olson, H. C., Paley, B., Mattson, S. N., Anderson, S. M., & Riley, E. P. (2016). Neurobehavioral disorder associated with prenatal alcohol exposure (ND-PAE): Proposed DSM-5 diagnosis. *Child Psychiatry and Human Development, 47*(2), 335–346.

Kiefer, Falk, et al. “Is Withdrawal-Induced Anxiety in Alcoholism Based on β-Endorphin Deficiency?” *Psychopharmacology*, vol. 162, no. 4, 25 June 2002, pp. 433–437., doi:10.1007/s00213-002-1118-y.

Koppers-Lalic, Danijela et al. “Virus-modified exosomes for targeted RNA delivery; a new approach in nanomedicine.” *Advanced drug delivery reviews* vol. 65,3 (2013): 348-56. doi:10.1016/j.addr.2012.07.006

Lamichhane, Tek N et al. “Exogenous DNA Loading into Extracellular Vesicles via Electroporation is Size-Dependent and Enables Limited Gene Delivery.” *Molecular pharmaceutics* vol. 12,10 (2015): 3650-7. doi:10.1021/acs.molpharmaceut.5b00364

Lehmann, P V. “The fate of T cells in the brain: veni, vidi, vici and veni, mori.” *The American journal of pathology* vol. 153,3 (1998): 677-80. doi:10.1016/S0002-9440(10)65609-X

Li XB, Zhang ZR, Schluesener HJ, Xu SQ. Role of exosomes in immune regulation. *J Cell Mol Med*. 2006;10(2):364–375. doi:10.1111/j.1582-4934.2006.tb00405.x

Marshall, S. Alex, et al. “Microglial Activation Is Not Equivalent to Neuroinflammation in Alcohol-Induced Neurodegeneration: The Importance of Microglia Phenotype.” *Neurobiology of Disease*, vol. 54, June 2013, pp. 239–251., doi:10.1016/j.nbd.2012.12.016.

May PA, Gossage JP, Kalberg WO, Robinson LK, Buckley D, Manning M, Hoyme HE. Prevalence and epidemiologic characteristics of FASD from various research methods with an emphasis on recent in-school studies. Dev Disabil Res Rev. 2009;15:176-92.

Méndez, Milagros, et al. “Acute Ethanol Administration Differentially Modulates μ Opioid Receptors in the Rat Meso-Accumbens and Mesocortical Pathways.” *Molecular Brain Research*, vol. 94, no. 1-2, 19 Oct. 2001, pp. 148–156., doi:10.1016/s0169-328x(01)00232-7.

Momen-Heravi, F., Bala, S., Kodys, K. *et al.* Exosomes derived from alcohol-treated hepatocytes horizontally transfer liver specific miRNA-122 and sensitize monocytes to LPS. *Sci Rep* **5,** 9991 (2015). https://doi.org/10.1038/srep09991

Murawski, N. J., Moore, E. M., Thomas, J. D., & Riley, E. P. (2015). Advances in diagnosis and treatment of fetal alcohol spectrum disorders: From animal models to human studies. *Alcohol Research: Current Reviews, 37*(1), 97–108.

Netea, Mihai G et al. “Trained immunity: A program of innate immune memory in health and disease.” *Science (New York, N.Y.)* vol. 352,6284 (2016): aaf1098. doi:10.1126/science.aaf1098

Pitt, J.M. , Kroemer, G. , Zitvogel, L. Extracellular vesicles: masters of intercellular communication and potential clinical interventions. J Clin Invest. 2016; 126(4): 1139–43.

Pastor, Raúl et al. “Involvement of the beta-endorphin neurons of the hypothalamic arcuate nucleus in ethanol-induced place preference conditioning in mice.” Alcoholism, clinical and experimental research vol. 35,11 (2011): 2019-29. doi:10.1111/j.1530-0277.2011.01553.

Pleet, Michelle L et al. “Autophagy, EVs, and Infections: A Perfect Question for a Perfect Time.” *Frontiers in cellular and infection microbiology* vol. 8 362. 18 Oct. 2018, doi:10.3389/fcimb.2018.00362

Raivich, Gennadij, et al. “Neuroglial Activation Repertoire in the Injured Brain: Graded Response, Molecular Mechanisms and Cues to Physiological Function.” *Brain Research Reviews*, vol. 30, no. 1, July 1999, pp. 77–105., doi:10.1016/s0165-0173(99)00007-7.

Sarkar, Dipak K. and Nadka I. Boyadjieva. “Role of Microglia in Ethanol’s Apoptotic Action on Hypothalamic Neuronal Cells in Primary Cultures.” *Alcoholism: Clinical and Experimental Research*, vol. 34, no. 11, 26 Oct. 2010, pp. 1835–1842., doi:10.1111/j.1530-0277.2010.01271.x.

Sarkar, Dipak K. and Nadka I. Boyadjieva. “Microglia play a role in ethanol-induced oxidative stress and apoptosis in developing hypothalamic neurons.” *Alcoholism, clinical and experimental research* vol. 37,2 (2013): 252-62. doi:10.1111/j.1530-0277.2012.01889.x

Shaaban A. Mousa, Mehdi Shakibaei, Nicolle Sitte, Michael Schäfer, Christoph Stein, Subcellular Pathways of β-Endorphin Synthesis, Processing, and Release from Immunocytes in Inflammatory Pain, Endocrinology, Volume 145, Issue 3, 1 March 2004, Pages 1331–1341, https://doi.org/10.1210/en.2003-1287

Shenoda, Botros B., and Seena K. Ajit. “Modulation of Immune Responses by Exosomes Derived from Antigen-Presenting Cells.” *Clinical Medicine Insights: Pathology*, Jan. 2016, doi:10.4137/CPath.S39925.

Sprouse-Blum, Adam S et al. “Understanding endorphins and their importance in pain management.” Hawaii medical journal vol. 69,3 (2010): 70-1.

Stein C. The Control of Pain in Peripheral Tissue by Opioids. N Engl J Med. 1995;332(25):1685–1690.

Tanabe S, Yamashita T. B lymphocytes: Crucial contributors to brain development and neurological diseases. *Neurosci Res*. 2019;139:37–41. doi:10.1016/j.neures.2018.07.002

Tian Y., Li S., Song J., Ji T., Zhu M., Anderson G.J., Wei J., Nie G. A doxorubicin delivery platform using engineered natural membrane vesicle exosomes for targeted tumor therapy. Biomaterials. 2014;35:2383–2390. doi: 10.1016/j.biomaterials.2013.11.083

Toda, Chitoku et al. "POMC Neurons: From Birth to Death." Annual review of physiology vol. 79 (2017): 209-236. doi:10.1146/annurev-physiol-022516-034110

Valadi, H. , Ekstrom, K. , Bossios, A. , Sjostrand, M. , Lee, J.J. , Lotvall, J.O. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol. Jun. 2007; 9(6): 654–9.

Vetreno R.P., Crews F.T. (2013) Innate Immune Signaling and Alcoholism. In: Cui C., Grandison L., Noronha A. (eds) Neural-Immune Interactions in Brain Function and Alcohol Related Disorders. Springer, Boston, MA

Wilhoit LF, Scott DA, Simecka BA. Fetal Alcohol Spectrum Disorders: Characteristics, Complications, and Treatment. *Community Ment Health J*. 2017;53(6):711–718. doi:10.1007/s10597-017-0104-0

Winkler, A., et al. "EFFECTS OF VOLUNTARY ETHANOL INGESTION ON THE POMC GENE EXPRESSION IN THE RAT PITUITARY AND ON THE PLASMA β-ENDORPHIN CONTENT." *Alcohol and Alcoholism*, vol. 30, no. 2, Mar. 1995, pp. 231–238., doi:10.1093/oxfordjournals.alcalc.a045719.

Xie, Yuanyuan, and Richard I Dorsky. "Development of the hypothalamus: conservation, modification and innovation." Development (Cambridge, England) vol. 144,9 (2017): 1588-1599. doi:10.1242/dev.139055

Yang, Tianzhi et al. "Exosome delivered anticancer drugs across the blood-brain barrier for brain cancer therapy in Danio rerio." *Pharmaceutical research* vol. 32,6 (2015): 2003-14. doi:10.1007/s11095-014-1593-y

Zaborowski, MikoŁaj P et al. "Extracellular Vesicles: Composition, Biological Relevance, and Methods of Study." *Bioscience* vol. 65,8 (2015): 783-797. doi:10.1093/biosci/biv084

Zalewska-Kaszubska, Jadwiga, and Elżbieta Czarnecka. "Deficit in Beta-Endorphin Peptide and Tendency to Alcohol Abuse." *Peptides*, vol. 26, no. 4, Apr. 2005, pp. 701–705., doi:10.1016/j.peptides.2004.11.010.

Zhao Z, Zlokovic BV. Remote control of BBB: A tale of exosomes and microRNA. *Cell Res*. 2017;27(7):849–850. doi:10.1038/cr.2017.71