FETAL ALCOHOL EXPOSURE INDUCES NEUROTOXIC EFFECTS ON β-ENDORPHIN NEURONS THROUGH MICROGLIAL ACTIVATION

By

Miguel Alexander Cabrera

A dissertation submitted to the

School of Graduate Studies

Rutgers, The State University of New Jersey

In partial fulfillment of the requirements

For the degree of

Doctor of Philosophy

Graduate Program in Endocrinology and Animal Biosciences

Written under the direction of

Dipak K. Sarkar

And approved by

_____

_____

_____

_____

New Brunswick, New Jersey

October, 2018

ABSTRACT OF DISSERTATION

Fetal Alcohol Exposure Induces Neurotoxic Effects On β-Endorphin Neurons Through Microglial Activation

By: Miguel Alexander Cabrera

Dissertation Director:

Dipak K. Sarkar

Fetal alcohol exposure has many detrimental effects on the developing brain and can cause fetal alcohol spectrum disorders (FASDs). Many FASDs patients show lifelong stress response abnormalities, demonstrated by an augmented response to stress hormones such as adrenocorticotropin and corticosterone (Lee et al., 2000), which are likely driven by alterations the hypothalamic-pituitary-adrenal (HPA) axis function (Zhang et al., 2005). Using a rat animal model, we have shown that postnatal ethanol exposure reduces the number and function of stress regulatory β-endorphin producing neurons in the hypothalamus, inducing a hyper-stress response (Sarkar et al., 2007; Logan et al., 2015). Microglia are one of the innate immune cells in the CNS and can be categorized as activated or ramified. Activated microglia are associated with an increase in proinflammatory responses and phagocytosis while ramified microglia are associated with maintaining homeostasis through dynamic communication, remodeling of neuronal

synapses, and surveying the environment (Bell-Temin et al., 2013). How β-endorphin neurons communicate with microglia to maintain normal homeostasis has yet to be addressed. β-endorphin can also bind to both mu- and delta-opioid receptors and may serve as a form of communication between β-endorphin neurons and microglia. Exosomes are small vesicles (30-150 nm) that play an important role in local and distant communication between cells. They carry unique cargo (proteins, mRNA, miRNA, and other non-coding RNAs) from the cells they originate from that can affect the recipient cell's homeostasis and induce apoptosis. Additionally, complement proteins, generally known for their role to opsonize foreign pathogens and support phagocytosis of dying cells may also play a role in ethanol-induced β-endorphin neuronal cell death. Here I demonstrate that ethanol-induced apoptosis of β-endorphin neurons is caused by activation of microglia to release proinflammatory cytokines, pro-apoptotic exosomes, and C1q. Furthermore, mu-opioid receptors activation is critical to ethanol-induced activation of microglia to induce apoptosis of β-endorphin neurons and antagonism of mu-opioid receptors attenuated the ethanol effect. Delta-opioid receptors antagonism did not have an effect on ethanol-induced β-endorphin neuronal cell death.

DEDICATION

Writing this dissertation and these past 5+ years of my Ph.D. research would not have been possible without the love and support from some incredible and important people in my life so I am dedicating this dissertation to them.

First, I would like to dedicate this dissertation to my wife and the love of my life, Ashley Nicole Motichka-Cabrera. Without her none of this would be possible. Ashley has supported me from the start of my gradate studies at Binghamton University; driving 3 hours each way just to spend time with me, watch our favorite shows, and cook some of the best meals I have ever tasted! Even now, after giving birth, she is doing her best to care for our son just so I can find the time to finish this dissertation. I will never be able to explain to her how much her love and support has meant to me but I will do my best to show her every day. I love her and I can't wait to raise our first son together!

Second, I would like to dedicate this dissertation to my son Matthew Nolan Cabrera. He was only born a few days ago but I already love him more than I could have ever imagined. He may have made writing this dissertation a little more difficult than I originally anticipated but I would not change a thing. This dissertation will serve as the bedrock for the future I plan to build for him and his mother. I cannot wait to see the man he grows up to be.

Next I would like to dedicate this dissertation to my parents, Gail Ann Nolan-Cabrera and Miguel Angel Cabrera. My mom's morning calls always started my day off the right way and her positivity helped me get through some of the more difficult times these last 5 years. She always knew when something was wrong and did her best to make me feel better. Even if I did not say it, I appreciated her support every time. She was the

"bestest" mom when I was younger and now she is the best mom and grandma. To my father, I am the man I am today because of him. I have adopted his stubbornness, sense of humor, calm demeanor, love of sports, and patience and I think those are my best qualities. I hope I can be as good a father to Matthew as he has been to me.

I would also like to dedicate this dissertation to my siblings, Natasha and Max. Natasha, my bookend, we grew up together sharing everything from bottles and cribs to notes in Anatomy (never forget how to pronounce gastrocnemius) & Molecular Biology and Biochemistry. She has continued to impress me ever since she learned to walk just so she could run away from me and run she has. Her dedication and perseverance have served as motivation for me to finish this dissertation. She's a great sister and I know she'll be a better aunt. One of the best parts of doing my Ph.D. research at Rutgers University was having the opportunity to spend 4 of those years with my little brother Max. Watching his growth from my little brother, who only wanted play Tibia and did not know how to run, into the mature, 2nd year medical student who still finds time to support causes he believes in and how to speak Spanish. This has been almost better than completing this dissertation.

Lastly I would like to dedicate this dissertation to Loki Heisenberg Cabrera. He was always waiting for me at the door when I had a rough day at the lab and made each evening better. I am happy we can finally get back to going to the dog park again!

ACKNOWLEDGEMENTS

In this section I would like to acknowledge some people whose support and mentorship significantly contributed to my dissertation and defined my career as a scientist.

First I would like to graciously acknowledge my committee chair and advisor, Dr. Dipak Sarkar. Allowing me to transfer into his lab from Binghamton University made this dissertation and the past 5 years possible. His mentorship and high expectations forced me to always strive to be better and allowed me to overcome many failed experiments during the first half of my dissertation. He also provided me with the freedom to take on multiple responsibilities and allowed to me grow into more than just a bench scientist. While I may not have found religion to make it through the tough times, as he once jokingly remarked, I found the support and leadership I needed to complete this dissertation.

I would like to thank my committee members: Drs. Nicholas T. Bello, Troy A. Roepke, and Long-Jun Wu for their support and mentorship throughout my time here and providing me the opportunity to earn my Ph.D.

Next I would like to thank all of the members of Dr. Sarkar's lab. To Shaima Amore Jabbar, she was the first person I met in Dr. Sarkar's lab and became one of my best friends. She taught me so many things from Iraqi culture to animal and laboratory techniques. I am grateful to have had the opportunity to get to know her, struggle through our Ph.D. research together, and teach her American culture. I'll admit that if I listened to her more often then I would have finished my Ph.D. in 4 years and published in Nature.

Ali Mosa Rashid Al-Yasari is one of the funniest people I have ever met and his friendship helped get me through these past years. His ability to complete your dissertation and still make his family a priority was something I always strived to match. In fact, that was one of the reasons I decided to stop spending 6-7 days a week in the lab and spend more time with my family. I will also never forget the time we spent in the animal facility with Kamil working on Ali's diabetes research.

Pallavi Shrivastava, Lucy Chastain, Sengottuvelan Murugan, and Omkaram Gangisetty, they have all served as a mentor to me at one point during my dissertation and I am grateful for everything they have taught me. Each of them has contributed to my dissertation in one way or another and I consider all of them as close friends. Thank you!

I would also like to thank Kamil Sochacki, Nadia Rachdaoui, Srinivas Gumudavelli, Sayani Mukherjee, Benedicte Rousseau, Eli Berger, and Ajay Palagani for their friendship and encouragement throughout my dissertation. Whether we have known each other for a long time or a short time, their presence made each day in the lab better.

Next, I would like to thank several members of the Animal Sciences department. First I would like to thank Carol Bagnell for her guidance and constant support throughout my graduate studies. She always had my best interest in mind and helped me finish my dissertation. Next, I would like to Jeannette Mullins for her friendship, help with anything I needed, and morning conversations. I would like to thank Damien Carroll-Debose for his assistance with every question or problem I had, and there were many of them. Thank you to Tory Gombosi for her positivity and assistance in numerous matters. She took the time to get to know me and made me feel welcome in the Animal Sciences department. Thank you to Kathy Manger for her help with all of the academic

issues and questions I had throughout my time here, as well as all of the pictures and gifts throughout the years. I would also like to thank the members of the graduate program, notably Mariana Soboya and Bryn Yeomans for their friendship and consult when it came thinking about non-academic careers.

I would like to thank everyone in the animal facility. Nancy, Jen, Matt, Amanda, and Jerry. All of you have made the years I spent in the animal facility enjoyable and saved my experiments more times then I would like to admit.

Lastly, I am grateful for all of the help iJOBS and Janet Alder have provided me this past year. I got a position at Target Health using the networking and interview skills I learned from the numerous events I attended. I do not know where I would be if I never attended all of the iJOBS events Janet organized. She truly helped me learn about all of the non-academic positions available to Ph.D.'s and provided some much needed mentorship.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AC	Adenylyl Cyclase
ACTH	Adrenocorticotropic Hormone
AD	Ad Libitum
ADH	Alcohol Dehydrogenase
AF	Alcohol-Fed
AKT	Protein Kinase B
ARBD	Alcohol-Related Birth Defects
ARND	Alcohol-Related Neurodevelopmental Disorder
Aβ	Amyloid β
cAMP	Cyclic Adenosine Monophosphate
CC3	Cleaved caspase-3
CNS	Central Nervous System
CRH	Corticotropin-Releasing Hormone
CSFR1	Colony Stimulating Factor Receptor 1
dbcAMP	Dibutyryl-cAMP
DOR	δ-Opioid Receptor
FAS	Fetal Alcohol Syndrome
FASDs	Fetal Acohol Spectrum Disorders
GPCR	G-Protein Coupled Receptor
GSH	Glutathione
HPA	Hypothalamic-Pituitary-Adrenal
IBA-1	Ionized Calcium-Binding Adapter Molecule 1
IL	Interleukin
JAK	Janus Kinase
LPS	Lipopolysaccharide
MAPK	Mitogen Activated Protein Kinase
MBH	Mediobasal Hypothalamus
MCP1	Monocyte Chemoattractant Protein 1
MCR	Melanocortin Receptor
MOR	μ-Opioid Receptor
MSH	α-Melanocyte Stimulating Hormone
ND-PAE	Neurodevelopmental Disorder Associated With Prenatal Alcohol Exposure
NFκB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NO	Nitric Oxide
NTD	Naltrindole
NTS	Nucleus Tractus Solitaries
NTX	Naltrexone
PC	Prohormone Convertases
PF	Pair-Fed

PI3K	Phosphatidylinositol-3 Kinase
PKA	Protein Kinase-A
PKC	Protein Kinase C
PND	Postnatal Day
POMC	Proopiomelanocortin
PVN	Paraventricular Nucleus of the Hypothalamus
ROS	Reactive Oxygen Species
SOD	Super Oxide Demutase
STAT	Signal Transducers and Activators of Transcription
TGF-β1	Tumor Growth Factor-β1
TLR-4	Toll-Like Receptor
TNF-α	Tumor Necrosis Factor-α

1. CHAPTER ONE: BACKGROUND INFORMATION

1.1 ALCOHOL USE IN WOMEN OF CHILDBEARING AGE

In 2008 it was estimated that over half of all pregnancies in the United States were unplanned (Finer et al., 2008). Moreover, most pregnant women are unaware they are pregnant until typically 4-6 weeks into their pregnancy (Green et al., 2013). Alcohol consumption is also common during this time period for women as demonstrated by a 2015 state-based survey. This survey reported that 51% of women of childbearing age (18-44) consumed any amount of alcohol and 16.9% engaged in a binge exposure (≥4 drinks in 2 hours, which produces a blood ethanol level of ≥0.08 g/dL) in the last 30 days (CDC, 2015: HHSH & USDA, 2015). Furthermore, it was estimated that 3.3 million women of childbearing age had consumed alcohol and engaged in sex without contraception (Green, 2013). The same study also found that 3 out of 4 women who consumed alcohol also wanted to get pregnant immediately (Green, 2013). These data demonstrate that it is very possible for a woman to be pregnant and expose her child to alcohol without her knowledge.

Alcohol consumption, however, can still occur throughout the pregnancy. A US-based study from 2015 reported that 10.2% of pregnant women reported to have consumed alcohol and 3.1% of pregnant women engaged in binge drinking in the last 30 days. Additionally, pregnant women consumed greater amounts of alcohol (4.6 drinks/binge) than their non-pregnant counterparts (3.1 drinks/binge). Interestingly, education level wasn't a deterrent as the highest prevalence of drinking was observed in college graduates aged 35-44 (Tan et al., 2015). Furthermore, it was estimated in 2013

that 5% of pregnant women in their second trimester and 4.4% of pregnant women in their third trimester consumed alcohol during in the last 30 days (SAMHSA, 2014). Alcohol consumption varies based on region and a study in the Netherlands found that in the second and third trimester, 13.5% and 11.6% of pregnant women consumed 1 alcohol drink per week, while 6.1% and 5.6% consumed 2 more drinks per week, respectfully (Guelinckx et al., 2011).

Alcohol consumption during pregnancy is a tremendous problem because it is a known teratogen and there is currently no known safe amount of alcohol during pregnancy. In 2005, the U.S. Surgeon General released an advisory on alcohol use during pregnancy urging women who are or may become pregnant to abstain from alcohol use (HHS, 2005). Alcohol consumption can be very harmful to the developing fetus and result in death, miscarriage, or the development of fetal alcohol spectrum disorders (FASDs).

1.2 FETAL ALCOHOL SPECTRUM DISORDERS

1.2.1 FASDs: EPIDEMIOLOGY AND DEFINITION

FASDs is an umbrella term used to group together a range of maladies induced by alcohol exposure during gestation. These maladies can be grouped into smaller categories typically 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). FAS is considered to be the most severe result of fetal alcohol exposure and is characterized by facial dysmophia, growth problems, and central nervous system abnormalities (CDC, 2018). Examples of central nervous system abnormalities are cognitive or developmental

deficits, executive functioning deficits, motor function delays, and problems with attention or hyperactivity (Bertrand et al., 2004). The CDC estimated that 0.2-1.5 infants are born with FAS for every 1000 live births (CDC, 2000). However, a more recent in-person assessment of children aged 7-9 years old in multiple U.S. communities found 6-9 children out of 1000 had FAS (CDC, 2015).

Patients with ARND or ARBD may exhibit intellectual and behavioral problems or organ specific maladies (heart, kidney, bones), respectfully (CDC, 2018), Patients with ND-PAE, a recent addition to the Diagnostic and Statistical Manual 5, have problems with thinking, memory, behavior, and normal day-to-day tasks such as issues with dressing or playing with peers (Hagan Jr. et al, 2016). Taking the entire spectrum of FASDs into consideration, the prevalence of FASDs has increased over the last 20 years in the U.S. Sampson et al. determined that the prevalence of FASDs was 1% in 1997 (Sampson et al., 1997). This number more than doubled a decade later with a prevalence of approximately 2-5% in the U.S. and Western Europe (May et al., 2009; May et al., 2014). Lastly, a recent report suggests that FASDs could be as prevalent as 9.8% in first-grade children using a weighted prevalence approach (May et al., 2018). Furthermore, the lifetime cost of FAS, one syndrome on the spectrum of FASDs, was estimated to be over $2 million per individual and $4-5.5 billion to the U.S. annually, however, these numbers are outdated and could be much higher due to inflation alone (Lupton et al., 2004; Sacks et al., 2010). Therefore, the prevalence and cost of FASDs is on the rise and needs to be addressed.

1.2.2 FETAL ETHANOL METABOLISM

In addition to maternal consumption of alcohol, metabolism of ethanol also affects the development of FASDs. First, it has been estimated that fetal blood ethanol levels reach an average of 83.5% of maternal levels within 1-2 hours after consumption (Waltman & Iniquez, 1972). Additionally, since ethanol is a small molecule, it can freely pass through the placenta into the fetus. In the placenta there is only one isoform of alcohol dehydrogenase (ADH), an enzyme that converts ethanol into acetaldehyde, which has a low affinity for ethanol and metabolizes it at a rate that is 3900-fold less than the adult liver (Karl et al., 1988; Burd et al., 2012). Moreover, the fetal liver has low amounts ADH and the P450 enzyme, which is required for CY2PE1 to function and metabolize ethanol, and therefore has a severely diminished ability (5-10% compared to an adult liver) to metabolize ethanol (Burd et al., 2012). Furthermore, excretion of ethanol, into the amniotic fluid through fetal breathing or urination, gets reabsorbed through swallowing of the amniotic fluid and reabsorption into the intramembranous pathway, recycling the teratogen and producing a longer exposure of ethanol to the fetus compared to the mother (Burd et al., 2012).

1.2.3 DEVELOPMENTAL TIMING OF ETHANOL EXPOSURE

Next, the developmental timing of ethanol exposure also plays an important role in determining the severity of FASDs. For example, the third trimester is a critical period in human fetal development marked by rapid brain growth and development termed “the brain growth spurt” (Dobbing, 1974). This time period is marked by neuronal differentiation, neurogenesis, myelination, and synaptogenesis (Sarkar et al., 2007). Exposure to ethanol during this critical period can be detrimental, resulting in decreased head circumference and brain weight, under development of the corpus callosum,

reduced volume of the cerebellum and basal ganglia, as well as learning and memory abnormalities, suggesting damage to the hippocampus in humans (Coles, 1994; HHS, PHS, NIH, & NIAAA, 2000). Studying this time period is important because it has been estimated in 2013 that 4.4% of pregnant women consumed alcohol during the third trimester in the US (SAMHSA, 2014). Moreover, 6% of pregnant women in the Netherlands reported that they consumed 1 alcoholic drink per week during the third trimester and 5.6% consumed at least 2 drinks per week (Guelinckx et al., 2011).

Lastly, determining the molecular mechanisms involved in producing FASDs requires animal models. The human third trimester is relative to the in first 10 days after birth in rats (Dobbing, 1974). During this time period in rats, postnatal ethanol exposure has been shown to produce similar effects in the brain, including loss of neurons in the hypothalamus, hippocampus, cortex, cerebellum, and corpus callosum, as well as decreases in head circumference and microcephaly (West et al., 1986; Kotkoskie & Norton, 1989; Chen et al., 2006). Currently there is no treatment for FASDs but early intervention can provide a promising avenue for treatment. Determining the molecular mechanisms through which fetal alcohol exposure induces these serious effects and finding ways to ameliorate or attenuate them is the current goal of my dissertation. Finally, the focus of this thesis will be on the mechanism through which fetal alcohol exposure induces apoptosis of β-endorphin producing neurons in the hypothalamus.

1.3 PROOPIOMELANOCORTIN

1.3.1 REGULATION OF POMC TRANSCRIPTION

Proopiomelanocortin (POMC) is a prohormone whose cleavage products are involved in the regulation of feeding and energy homeostasis, stress, heart rate and blood

pressure, and sexual function (Martin & MacIntyre, 2004; Cone, 2005; Mountjoy, 2015). The POMC prohorome is transcribed from a single gene, which consists of 3 exons and 2 introns. This gene is represented on chromosome 2 in humans and chromosome 6 in rats (Overwatch et al., 1981; Pintar et al., 1984; Ogino et al., 2003). Exon 1 codes for an untranslated sequence, exon 2 codes for the signal peptide and the first amino acids of the N-terminal peptide (NT), and exon 3 codes for most of the translated mRNA, including the C-terminal part of the NT, adrenocorticotropic hormone (ACTH), and β-lipotrophin (β-LPH) (Raffin-Sanson et al., 2003). Determination of which protein is produced is governed by posttranslational processing of POMC and is tissue specific. POMC mRNA is expressed in the arcuate nucleus of the hypothalamus, nucleus tractus solitaries (NTS), anterior pituitary gland, adrenal gland, fat, pancreas, skin, immune system, and several other tissues (Yeo et al., 2000; Lundberg et al., 2014; Toda et al., 2017). While POMC has numerous functions in the tissues it is expressed, this review will focus primarily on the anterior pituitary and the arcuate nucleus of the hypothalamus.

In the pituitary gland, POMC is expressed only in hormone secreting cells called corticotropes in the anterior pituitary and melanotropes in the intermediate lobe (Sarkar, 2015). It should be noted that humans have a prominent intermediate lobe during fetal development but in adults it has no discrete anatomical location (McNicol, 1986). POMC expression in the anterior pituitary is positively regulated by corticotropin-releasing hormone (CRH) and negatively regulated by glucocorticoids (Roberts et al., 1979; Loeffler et al., 1985, Roberts et al., 1987). CRH acts through its G-protein coupled receptor (GPCR) to activate adenylyl cyclase (AC), increase cyclic adenosine monophosphate (cAMP), and activate protein kinase-A (PKA). PKA then phosphorylates

PCRH-RE, a transcription factor, to induce transcription of POMC (Sarkar, 2015). CRH can also promote transcription through activation of c-fos, FosB, JunB, and binding to the POMC AP-1 promoter site (Sarkar, 2015). Glucocorticoids, on the other hand, suppress POMC transcription through the binding of a negative glucocorticoid response element to the POMC promoter. Binding of a glucocorticoid to a monomeric glucocorticoid response element leads to the binding of another monomer, which dimerizes to the other side of the DNA helix and partially blocks the CCAAT box of the POMC promoter (Sarkar, 2015). POMC is also regulated by insulin and leptin (D'Agostino & Diano, 2010). Insulin can bind to insulin receptors on POMC neurons and activate phosphatidylinositol-3 kinase (PI3K). Once activated, PI3K phosphorylates membrane lipid phosphatidylinositol-4,5-bisphosphate to phosphatidylinositol-3,4,5-trisphosphate (PIP3). PIP3 then binds to phosphoinositide-dependent protein kinase 1 (PDK1) and phosphorylates protein kinase B (AKT). Phosphorylated AKT then phosphorylates a transcription factor called forkhead box-containing transcription factor of the O subfamily type 1 (FOXO1), which is an inhibitor of POMC transcription, and phosphorylation removes FOXO1 from the POMC promoter region (Kitamura, 2006; D'Agostino & Diano, 2010). Interestingly, activation of PI3K can also induce the opening of adenosine triphosphate (ATP)-sensitive potassium channels, depolarize POMC neurons, and augment firing of these neurons (Shyng & Nichols, 1998; Parton et al., 2007). Leptin binds to leptin receptors on POMC neurons and activates the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway, particularly STAT3 (Vaisse et al., 1996). Once activated, STAT3 can recruit histone acetylases to the POMC promoter and induce POMC transcription (Kitamura, 2006).

Taken together, these mechanisms demonstrate how POMC transcription can be regulated; however, POMC also undergoes posttranslational modifications.

1.3.2 POSTTRANSLATIONAL PROCESSING OF POMC

The posttranslational processing of POMC begins in the Golgi bodies. In the Golgi bodies, the 32 kilodalton prohormone receives modifications that target it to specific secretory granules where it will be processed and stored before being released by exocytosis (Cool et al., 1997; D'Agostino & Diano, 2010). POMC is cleaved within these granules by a family of serine proteases called prohormone convertases (PC) (Pritchard, et al., 2002). In the anterior pituitary, PC1 but not PC2 is present. PC1 cleaves POMC into Pro-ACTH, signal peptide (SP), and β-LPH. Pro-ACTH is further cleaved into ACTH, NT, and joining peptide (JP) while β-LPH can be cleaved to produce small amounts of gamma-LPH (γ-PLH) and β-endorphin (Bertanga, 1988; Zhou et al., 1993; Pritchard et al., 2002; Bicknell, 2008). Alternatively, the intermediate lobe and the hypothalamus contain both PC1 and PC2. This allows for more extensive processing of POMC products than in the anterior pituitary, producing smaller peptides (Mains et al., 1989; Bicknell, 2008). In these regions, ACTH is cleaved into α-melanocyte stimulating hormone (α-MSH) and corticotropin-like intermediate protein (CLIP). β-LPH is cleaved into γ-PLH and β-endorphin (Deakin et al., 1980; Bicknell, 2008). One important difference between the intermediate lobe and the hypothalamus is that in the intermediated lobe β-endorphin is further cleaved at the c-terminus, which eliminates its potency for analgesia. This does not occur in the hypothalamus (Deakin et al., 1980; Bicknell, 2008). Therefore, in the anterior pituitary, POMC posttranslational modifications lead primarily to the production ACTH while neurons in the hypothalamus

can produce smaller peptides such as β-endorphin and α-MSH. Next I will briefly describe the action of the aforementioned POMC cleavage products (ACTH, α-MSH, β-endorphin).

ACTH is a key component of the hypothalamic-pituitary-adrenal axis because it responds to CRH following a stressor and induces the production of glucocorticoids to respond to the stressor (Tsigosa & Chrousos, 2002). α-MSH binds to melanocortin receptors (MCR) in numerous peripheral tissues and regulates skin pigmentation, adrenal steroidogenesis, and thermoregulation (Chen et al., 1993; Clark et al., 1993; Robbins et al., 1993). In the hypothalamus, α-MSH binds to MC3R and MC4R to regulate food intake and energy homeostasis. Finally, β-endorphin is an endogenous opioid peptide that preferentially binds to mu-opioid receptors (MOR) over delta-opioid receptors (DOR) and is involved in regulating stress, pain, and reward (Koneru et al., 2009). Focusing on the stress axis and the role β-endorphin plays in its regulation will be the focus of the next section.

1.4 THE HPA AXIS AND STRESS

1.4.1 HPA AXIS

The HPA axis is a neuroendocrine system that operates on a negative feedback loop to regulate the body's response to stress. Some examples of stress are systemic stressors (physical or metabolic) such as changes in blood pressure, pain, and hypoglycemia, or psychogenic stressors such as emotional distress (Aguilera & Liu, 2012). Systemic stressor stressors utilize ascending pathways originating in the NTS and the ventrolateral medulla. Adrenergic (A1 and C1) and noradrenergic (A2 and C2) fibers from the NTS and ventrolateral medulla, respectively, synapse onto CRH neurons in the

paraventricular nucleus of the hypothalamus (PVN) and activate α-adrenergic receptors, which are coupled to guanyl nucleotide binding protein q/11 (Gq11) and phospholipase C (PLC) (Kiss and Aguilera, 1992; Herman et al., 2003). Activation of PLC induces the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP2) into diacyl glycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). These secondary messengers induce the activation of protein kinase C (PKC) and the release of intracellular Ca^{2+}, which induces CRH release from the PVN. Additionally, glutamate signaling through NMDA or GluR5 can also increase intracellular Ca^{2+} and induce CRH release (Aguilera & Liu, 2012). PKC activation as well as activation of PKA, calcium calmodulin dependent protein kinase, and mitogen activated protein kinase (MAPK), can phosphorylate and activate cAMP responsive element binding protein and induce transcription of CRH (Liu et al., 2008; Aguilera & Liu, 2012). Therefore, stress not only induces the release of CRH from the PVN but also increases the transcription of CRH to respond to a stressor.

Once activated, CRH is released into the hypophyseal portal system where it binds to the CRH receptor 1 and stimulates the transcription and release of ACTH from corticotrope cells in the anterior pituitary (Oyola & Handa, 2017). ACTH enters the bloodstream and binds to the MC2R in the zona fasciculate of the adrenal cortex (Hadley & Haskell-Luevano, 1999). MC2R is a GPCR and binding of ACTH produces a confirmation shift in the GPCR resulting in the activation of AC. AC induces the conversion of ATP to cAMP, which then phosphorylates PKA. (Grahame-Smith et al., 1967; Spät et al., 2016). PKA phosphorylates and activates hormone-sensitive lipase, which cleaves intracellular lipid droplets to produce free cholesterol. PKA also activates the steroidogenic acute regulatory protein that transfers the free cholesterol into the

mitochondria (Kim et al., 1997; Holm et al., 2000). Furthermore, PKA phosphorylates L-type Ca^{2+} channels and produces an influx of extracellular Ca^{2+} that activates PLC (Gallo-Payet et al., 1996). The PKC signaling mechanism results in the generation of IP_3 and subsequent release of intracellular Ca^{2+}. Calcium activates three Ca^{2+}-dependent mitochondrial dehydrogenases in the inner mitochondrial membrane, induces the production NADPH, and serves as an important cofactor in glucocorticoid synthesis (Spät et al., 2016). Glucocorticoids then enter the bloodstream and coordinate the stress response, resulting in gluconeogenesis, increased arousal, immunosuppression, elevated heart rate, vasodilation, and modified learning and memory (Stephens, 2012). Meanwhile, glucocorticoids can bind to CRH neurons and corticotropes and inhibit the activation of the HPA axis in a negative feedback manner. β-endorphin, a cleavage product of POMC can also inhibit CRH activation. β-endorphin neurons send projections to CRH neurons in the PVN and can inhibit the release of CRH (O'Connor et al., 2000). This occurs through binding of β-endorphin to MORs on CRH neurons. MORs are coupled to inhibitory GPCRs and activation of these receptors induces G_i signaling. G_i inhibits the activity of AC and reduces the production of cAMP. Reductions in cAMP diminish Na^+ influx and suppress neuronal excitability by preventing the opening of cyclic nucleotide-gated ion channels. Then the βγ subunits of the inhibitory GPCR inhibit T-type calcium channels and prevent Ca^{2+} influx and depolarization. The βγ subunits also activate the G-protein inwardly rectifying potassium (GIRK) channels. Activation of GIRK channels induces neuronal hyperpolarization through K^+ efflux (Chan et al., 2017). In summary, the HPA axis is an endocrine system that produces a response to a stressor. CRH activates the production of ACTH, which in turn induces the production and release

of glucocorticoids. Glucocorticoids carry out the response to a stressor as well as negatively regulate HPA axis to prevent hyperactivation. In addition, β-endorphin also plays a critical role in the regulation of the HPA axis. Despite these numerous regulators of the HPA axis, it is very susceptible to programming during fetal development.

1.4.2 DYSREGULATION OF THE HPA AXIS

Hyperactivation, such as with repetitive stressors or the loss of a negative regulator can reprogram the HPA axis such that it becomes hyperresponsive to stressors throughout life (Zhang et al., 2005). Many researchers have, in fact, demonstrated this effect with repetitive stressor exposure during the prenatal developmental period, producing lifelong hyperresponsiveness to stress. (Weinberg et al., 2008). Takahashi et al. found that prenatally stressed rats exhibited augmented ACTH and corticosterone levels following a foot shock stress paradigm at postnatal days (PND) 7, 14, and 21 Takahashi et al., 1991). Henry et al. found elevated levels of corticosterone in the plasma of prenatally stress exposed male rats after exposure to a novel object at PND 3, 7, and 90. Interestingly, they also found that density of hippocampal type I and type II corticosteroid receptors were reduced in PND 21 and 90 male rats (Henry, et al., 1994). Lastly, Mueller & Bale found decreased levels of *Crf* and glucocorticoid expression in the hypothalamus, along with increased methylation at their promoter regions (Mueller & Bale, 2013). Taken together, these experiments show that prenatal stressors can produce lifelong hyperstress responses to acute physiological and psychogenic stressor.

Our lab has shown in rats and mice that chronic ethanol exposure induces apoptosis of β-endorphin neurons or reduces POMC expression in the arcuate nucleus following prenatal, postnatal, and preconception ethanol exposure (Sarkar et al., 2007;

Agapito, et al., 2013; Bekdash et al., 2013; Jabbar et al., 2016, Shrivastava et al., 2017). Moreover, our lab has demonstrated that the reduction of BEP neurons following fetal ethanol exposure contributes to HPA hyperresponsiveness to immune challenge and behavioral stressor (open-field maze and elevated plus maze) in adulthood. Critically, this anxiety and hyperresponsiveness to stress phenotype was eliminated in rats that received β-endorphin transplantation into the PVN (Logan et al., 2015). This suggests that the loss of β-endorphin neurons leads to a hyperresponsiveness to stress. This is consistent with the expected outcome of removing a negative regulator of the HPA axis. With fewer β-endorphin neurons to inhibit HPA activation, the HPA axis may be programmed to respond in a hyperresponsive manner when activated. Importantly, ethanol was the primary toxicant that was used to induce apoptosis of β-endorphin neurons, so the next section will discuss potential mechanisms through which ethanol can induce apoptosis.

1.5 ETHANOL-INDUCED APOPTOSIS OF β-ENDORPHIN NEURONS

1.5.1 IN VITRO MECHANISMS FROM PRIMARY HYPOTHALAMIC CULTURE

In order to manage and prevent ethanol-induced apoptosis of β-endorphin neurons our lab has been trying to elucidate the mechanisms involved. One mechanism through which ethanol induces apoptosis was demonstrated by Chen et al. in a fetal hypothalamic culture. Here they showed that chronic ethanol exposure (2 and 4 days) increased nucleosome activity, decreased β-endorphin count, increased TUNEL-positive β-endorphin neurons, and increased caspase-3 activity. Caspase-3 is a potent apoptotic protein that is involved in chromatin condensation and DNA fragmentation (Porter & Jänicke, 1999). Importantly, administration of Ac-DEVD-CHO, a potent caspase-3 inhibitor, reduced ethanol-induced loss of β-endorphin neurons and TUNEL-positive β-

endorphin neurons. Furthermore, Chen et al. also found reductions in cAMP protein and AC-7 and -8 mRNA following chronic ethanol exposure. Moreover, these effects, as well as increased TUNEL-positive β-endorphin neurons, were also found following administration of an AC inhibitor called dideoxy-adenosine (DDA). The effect of DDA treatment was potentiated by ethanol co-administration at a low dose. The cAMP analog dibutyryl-cAMP (dbcAMP), when treated along with ethanol, completely blocked ethanol-induced death of β-endorphin neurons. In addition, chronic ethanol exposure, as well as DDA treatment, produced increased tumor growth factor-β1 (TGF-β1) protein and mRNA expression in a hypothalamic culture, which was blocked by co-administration of ethanol and dbcAMP. Moreover, TGF-β1 treatment dose-dependently increased apoptosis of β-endorphin neurons and this effect did not increase when co-administered with ethanol or DDA, suggesting that ethanol, DDA, and TGF-β1 share a common pathway to induce apoptosis of β-endorphin neurons. Anti-TGF-β1 antibodies attenuated this effect (Chen et al., 2006a). Importantly, TGF-β1 is known to induce apoptosis via Bcl-2 class of mitochondrial proteins (Francis et al., 2000). Lastly, all three (ethanol, DDA, and TGF-β1) treatments separately increased mRNA expression of apoptotic markers TGF-β1, Bcl-xs, Bax, Bak, and caspase-3, and decreased anti-apoptotic marker Bcl-2, only TGF-β1 exposure decreased Bcl-xl (Chen et al., 2006a). When activated, Bax can bind to the endoplasmic reticulum and mitochondria to form pores that release Ca^{2+} ions and cytochrome c, respectively. Bak can oligomerize within mitochondria and increase the permeabilization of the mitochondrial outer membrane and release cytochrome c. Released cytochrome c binds to Apaf-1 and induces a signaling cascade that results in the activation of caspase-3. Bcl-2 and Bcl-xl can prevent this by

binding to Bak and Bax, respectfully, and inactivating them (Sánchez-Capelo, 2005). Taken together, these data suggest that a potential mechanism through which ethanol exposure induces apoptosis of β–endorphin neurons is via the reduction of cAMP and activation of a TGF-β1-dependent mechanism for apoptosis.

1.5.2 IN VIVO MECHANISMS

Two *in vivo* experiments built on the findings of Chen et al. regarding the mechanism for ethanol-induced apoptosis of β-endorphin neurons. Sarkar et al. found that postnatal ethanol exposure increased TUNEL-positive β-endorphin neurons, TGF-β1, Bcl-xs, and Bad mRNA expression and decreased the mRNA expression of POMC, AC6, AC8, and Bcl-2 in the arcuate nucleus. Furthermore, the loss of β-endorphin neurons persisted into the juvenile period, well after the cessation of ethanol exposure. Interestingly, they also found that CRH mRNA was increased following exposure to lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria and a known endotoxin, in the juvenile period and this increase could not be attenuated by BEP administration (Sarkar et al., 2007). Kuhn et al. demonstrated that ethanol-induced production of TGF-β1 induces apoptosis via regulation of cyclin dependent kinases (cdk). In this experiment, postnatal ethanol exposure reduced Cdk-4 and Cyclin D3 and increased the cdk inhibitor P27/kip in the hypothalamus (Kuhn & Sarkar, 2008). Cdks are known to regulate the cell cycle and dysregulation of these kinase activities through TGF-β1 has been shown to induce apoptosis via altered phosphorylation of the transcriptional repressor retinoblastoma protein (Rb) (Oh et al., 2000). Rb represses E2Fs which can induce apoptosis through the transcription of the Bcl class of apoptotic proteins (Callagan et al., 1999; Saavadra et al., 2002; Kuhn & Sarkar,

2008). However, Kuhn et al. found that chronic ethanol exposure decreased phosphorylation of Rb, expression of E2F1, and increased expression of E3F5 (Kuhn & Sarkar, 2008). Decreased phosphorylation of RB has been shown to induce apoptosis in immature neurons through decreased expression of critical growth factors, such as insulin growth factor (Porcu et al., 1994; Day et al., 1997, Yu et al., 2001). Lastly, chronic ethanol exposure increased the expression of pro-apoptotic factors Bad and Bcl-xs and decreased anti-apoptotic factor Bcl-2, in congruence with previous findings by Chen et al. and Sarkar et al. (Chen et al., 2006a; Sarkar et al., 2007; Kuhn & Sarkar, 2008). These data build on previously described data to demonstrate that chronic ethanol exposure induces apoptosis of β-endorphin neurons through TGF-β1 dependent pathways.

1.5.3 ROLE FOR ROS IN ETHANOL-INDUCED APOPTOSIS OF β-ENDORPHIN NEURONS

Ethanol exposure in a mixed hypothalamic neuronal culture increased superoxide anion (O_2^-), reactive oxygen species (ROS), and nitrite (Boyadjieva & Sarkar, 2013). ROS can induce apoptosis through permeabilization of the mitochondrial outer membrane to release cytochrome c or through chemical reactions with key cell macromolecules, namely, phospholipids, proteins and DNA, which may lead to cellular dysfunction (Yu et al., 2001). Augmentation of free radicals and ROS was further exacerbated by ethanol-induced attenuation of endogenous antioxidant glutathione (GSH), as well as the enzymes involved in generating antioxidants such as catalase, super oxide demutase (SOD), and GSH-px. Additionally, the effects of ethanol exposure on apoptosis and oxidative stress were promoted by the presence of microglia (Boyadjieva & Sarkar, 2013). In primary microglia culture ethanol activated microglia augmented the

production tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), macrophage inflammatory protein 1-alpha (MIP-1α), and macrophage inflammatory protein 2-alpha (MIP-2). Also, the conditioned media of activated microglia induced apoptosis of cultured hypothalamic neurons. Immunoneutralization of TNF-α reduced the ability of ethanol-activated microglia to induce apoptosis of hypothalamic neurons (Boyadjieva & Sarkar, 2011). In summary, these data suggests that ethanol-induced apoptosis of β-endorphin neurons may involve ROS, TGF-β1, and microglia. In the next section I will focus more on microglia and the role they play in ethanol-induced apoptosis and CNS communication.

Table 1. Summary of studies investigating ethanol-induced apoptosis of hypothalamic neurons utilizing *in vitro* and *in vivo* models

	Ethanol Exposure Model	Dose (mg/dl or mM)	Brain Region/Culture	Ethanol Effect	Neuronal Apoptosis	Reference
1	*In vitro* - Chronic Exposure (2, 4 days)	50, 100, 150 mM	Primary Hypothalamic Culture	↑Cleaved Caspase 3, TGF-β1, Bcl-xs, Bax, Bac ↓Adenylyl Cyclase, Bcl-2, Bcl-xl	↑β-Endorphin apoptosis	Chen et al., 2006a
2	*In vivo* – Chronic Intermittent Exposure (5 Days) in Postnatal (PND 3-7) Sprague Dawley Rats	2.5 g/kg, 200-250 mg/dl	Arcuate Nucleus	↑TGF-β1, Bcl-xs, & Bad ↓POMC, Adenylyl cyclase, Bcl-2	↑β-Endorphin apoptosis	Sarkar et al., 2007
3	*In vivo* - Chronic Intermittent Exposure (5 Days) in Postnatal (PND 2-6) Sprague Dawley Rats	2.5 g/kg, 200-250 mg/dl	Arcuate Nucleus	↑TGF-β1, Cdk inhibitor p27/kip, Bcl-xs ↓Phosphorylated Rb, Cdk4, Cyclin D3, Bcl-2	↑β-Endorphin apoptosis	Kuhn & Sarkar, 2008
4	*In vitro* – Chronic Exposure (2, 4 days)	25, 50, 100 mM	Primary Hypothalamic (MBH) Culture & Primary Microglia Culture	Microglia ↑OX-6, TNF-α, MIP-1, IL-6 in media Ethanol-activated microglia conditioned media induced apoptosis of MBH neurons	↑MBH neuronal apoptosis	Boyadjieva & Sarkar, 2010
5	*In vitro* – Chronic Exposure (2, 4 days)	25, 50, 100 mM	Primary MBH Culture	↑O_2^-, ROS, Nitrite ↓GSH, GHS-Px SOD, Catalase *Effect further exacerbated by ethanol-activated microglia conditioned media	↑MBH neuronal apoptosis	Boyadjieva & Sarkar, 2013

↑, Increase in gene or protein expression; ↓, Decrease in gene or protein expression; PND, Postnatal day; MBH, Mediobasal hypothalamus

1.6 MICROGLIA

1.6.1 OVERVIEW OF MICROGLIA FUNCTION

Microglia are one of the innate immune cells of the brain and represent about 10% of the brain cell population. They originate from the yolk sac and enter the embryo at embryo day 8 (E8), surround the exterior of the neuroepithelium by E9.5, and are found in the neuroepithelium by E10.5. Microglia continue to populate the brain through the first 2 postnatal weeks, resulting in the generation of about 95% of their total population (Alliot et al., 1999; Ransohoff, 2011).

Microglia behave similarly to macrophages, as they are the surveyors and protectors of the CNS. In a healthy CNS, microglia tend to be in a ramified state where they actively scan the surrounding environment with their mobile processes, which is critical to the maintenance of CNS homeostasis (Kettenmann et al., 2011). Interestingly, it is suggested that the territory individual microglia occupy does not overlap with adjacent microglia (Ransohoff & Perry, 2009). Microglia are actively involved in maintaining neuronal synapses, promoting neurogenesis through the release of neurotrophic factors, and supporting learning and memory (Wake et al., 2009; Kettenmann et al., 2011; Paolicelli et al., 2011; Tremblay et al., 2011; London et al., 2013). If microglial processes detect an insult or if there is an injury to the CNS, microglia have the ability to shift their phenotype to an M1 phenotype. The M1 phenotype is generally associated with an increase in the size of the microglia soma and withdrawal of the processes, resembling an amoeboid appearance. Additionally these microglia also exhibit increased motility, upregulation of activation markers CD14, F4/80, CD11b, and CD86, production of proinflammatory cytokines such as IL-1β, TNF-

α, nitric oxide (NO), and ROS, the ability to proliferate, and phagocytosis of damaged cells or debris (Wang et al., 2002; Kettenmann et al., 2011; Chhor et al., 2013; Chastain & Sarkar, 2014; Walker & Lue, 2015; Tang & Le, 2016; Shrivastava et al., 2017). After M1 microglia eliminate the insult they can transition back to an M2 phenotype and provide anti-inflammatory and repair mechanisms to heal neurons and return to homeostasis. M2 microglia characteristics differs from M1 as they exhibit longer and thinner processes, smaller soma, produce anti-inflammatory cytokines and trohpic factors including IL-4, IL-13, IL-10, TGF-β, IGF1, and BDNF, and upregulate certain cell surface markers such as CD200R, CD33 and TREM-2 (Walker & Lue, 2015; Tang & Le, 2016). However, this transition to M2 can be halted by chronic activation of microglia via a toxicant or insult. Examples of this can be observed in Parkinson's disease, Alzheimer's disease, multiple sclerosis, and chronic alcohol consumption (Wu et al., 2002; Yan et al., 2003; Wilms et al., 2010; Kettenmann et al., 2011). In summary, microglia are vital regulators of neuronal homeostasis but chronic activation can lead to dysregulation and cell death. The next section will cover the role ethanol plays in activating microglia.

1.6.2 ETHANOL-INDUCED ACTIVATION OF MICROGLIA

1.6.2.1 ETHANOL-INDUCED UPREGULATION OF MICROGLIAL ACTIVATION MARKERS

Ethanol has been shown to activate microglia in a myriad of ways and clinical examples of microglial activation can be found in the analysis of postmortem brains of alcoholics. These studies demonstrated activation of microglia through upregulation of microglial markers such as ionized calcium-binding adapter molecule 1 (IBA-1), CD11b, and toll-like repceptor-7, as well as proinflammatory cytokines including monocyte

chemoattractant protein 1 (MCP1) and high mobility group box 1 (HMGB1), and NADPH oxidase (NOX) expression (He & Crews, 2008; Qin & Crews, 2012; Coleman et al., 2017).

In vivo studies of ethanol exposure have also shown microglial activation and proliferation as soon as 2 days after an adult binge exposure in the hippocampus (dentate gyrus, CA1, CA2/3, and hilus) and cortex (Nixon et al., 2008; Kelso et al., 2011). These changes were demonstrated by increased staining of ED1, a lysosomal antigen expressed in phagocytes and brdU, a maker for proliferation. While the previous data found changes in microglial activation following high doses of ethanol exposure (blood ethanol level >300 mg/dL), studies that produced a lower level of blood ethanol also found changes in microglial activation. Zhao et al., found increases in microglial activation, demonstrated by increased Cd11b staining, in the hippocampus only 1 day after an adult binge exposure that coincided with increased IL-1β and TNF-α protein expression and decreased neuronal cell number and dendritic spine density (Zhao et al., 2013). Importantly, these changes were observed with a peak blood ethanol concentration of 170 mg/dL. The developmental timing of ethanol exposure may also play a role in the degree of microglial activation. McClain et al. exposed adolescent rats to a binge model of ethanol exposure and found increases in microglial proliferation but no change in ED1 staining or inflammatory cytokine production. The authors suggested that this ethanol paradigm in adolescents only partially activated microglia (McClain et al., 2011). However, it should be noted that the blood ethanol concentrations observed in these animals were 300-400 mg/dL so that could have also been a reason why microglia weren't as fully activated as observed in Zhao et al. Fetal ethanol exposure has been

shown to also activate microglia in both acute and chronic models. Saito et al. found that after one exposure to ethanol in PND 7 microglia expressed thicker processes and colocalized with GM2 ganglioside, which is upregulated during apoptotic neurdegeneration (Saito et al., 2012). Lastly, Shrivastava et al. found that 5 days of postnatal ethanol exposure increased IBA-1 expression of microglia in the arcuate nucleus, as well as the number of fully amoeboid and partially amoeboid microglia. These changes coincided with augmented inflammatory signals and decreased POMC neurons and spine density in the hypothalamus. Furthermore, microglia isolated from the hypothalamus expressed augmented IBA-1, MOR, toll-like receptor (TLR-4), and TLR-4 dependent signaling molecules, suggesting a pronounced activation of microglia (Shrivastava et al., 2017). These data taken together demonstrate that chronic and acute ethanol exposures have the potential to activate microglia in humans and animals. Dose and developmental timing may also play a role in the degree of microglial activation. Next I will explain some of the molecular mechanism underpinning ethanol-induced activation of microglia.

1.6.2.2 TLR-4 AND NF-κB

Animal models of microglial activation have also been shown to involve TLR-4. Ethanol activates TLR-4 and specifically upregulates downstream signaling molecules nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells (NF-κB), MAPKs, and MyD88-independent pathways to trigger the production of proinflammatory cytokines interleukin-1 beta (IL-1β), TNF-α, and nitric oxide to promote cellular apoptosis (Fernandez-Lizarbe et al., 2009). NF-κB signaling is critically important in this TLR-4 cascade because NF-κB regulates the transcription of cytokines and chemokines and

functions in conjunction with other transcription factors for proinflammatory genes such as AP-1 and the nuclear factor of IL-6 (Cone, 2001). These effects were eliminated in TLR-4 knockout mice (Fernandez-Lizarbe et al., 2009). Ethanol has also been shown to augment the release of HMGB1, a known ligand for TLR-4, and the receptor for advanced glycation end products (RAGE1), which was also increased following intermittent ethanol exposure. Activation of RAGE1 by HMGB1, similar to TLR-4, results in the upregulation of NF-κB and proinflammatory cytokine release (Vetreno et al., 2013). Moreover, acute and chronic ethanol exposure has been found to activate NF-κB, produce proinflammatory cytokines, and further activate TLR-4 (Yang et al., 2014). These data explain how ethanol can activate microglia through TLR-4 dependent mechanisms, including NFκB. Next I will discuss the role ROS play in ethanol-induced activation of microglia.

1.6.2.3 REACTIVE OXYGEN SPECIES

Ethanol exposure can also indirectly activate microglia through the induction of neuronal apoptosis. In neurons, metabolism of ethanol via CYP2E1 or its metabolites via NOX and nitric oxide synthase has been shown to produce ROS with the ability to induce oxidative damage and neurodegeneration (Haorah et al., 2009). Additionally, NOX is the major enzyme for the production of ROS in microglia and NOX can be activated by ethanol-derived acetaldehyde (Block, 2008; Alikunju et al., 2011; Choi et al., 2012). Other studies have demonstrated that ethanol exposure increases ROS and the mRNA expression of Duox1, a NOX catalytic subunit and NOX regulatory subunits including p22phox, p67phox, NOX activator 1, and NOX organizer 1. NOX activity was also found to elevated and co-treatment of DPI (a NOX inhibitor) significantly diminished ROS

production (Dong et al., 2010). Additionally, ethanol exposure can reduce the concentration of endogenous antioxidants, which are inhibitors of ROS and oxidative stress (Henderson et al., 1995; Boyadjieva & Sarkar, 2013). Depletion of antioxidants coupled with a rise in ROS can induce neuronal apoptosis and activate microglia (Chastain & Sarkar, 2014). Importantly, inactivation of microglia through administration of minocycline (a tetracycline known to inhibit microglial activation) has been shown to prevent ethanol-induced neurodegeneration and inflammation (Wu et al., 2011; Qin et al., 2012).

Table 2. Summary of studies investigating ethanol-induced activation of microglia utilizing human postmortem brains, *in vitro* and *in vivo* models.

		Ethanol Exposure Model	Dose (mg/dl or mM)	Brain Region/Culture	Ethanol Effect	Neuronal Apoptosis	Reference
Microglial Activation in Human Postmortem Alcoholic Brains	1	Human Postmortem Brains (Alcoholic vs. Moderate Drinking Controls)	N/A	Multiple	↑MCP-1, IBA-1 (Cingulate Cortex), & GLUT 5 (Cingulate, VTA, & Midbrain)	N/A	He & Crews, 2008
	2	Human Postmortem Brains (Alcoholic vs. Moderate Drinking Controls)	N/A	Orbital Frontal Cortex (OFC)	↑Fluoro-Jade B colocalization on neurons & NOX subunit (gp91) colocalization on microglia	↑Neuronal apoptosis	Qin & Crews, 2012
	3	Human Postmortem Brains (Alcoholic vs. Moderate Drinking Controls)	N/A	Hippocampus	↑TLR7, HMGB1, & CD11b	N/A	Coleman et al., 2017
Ethanol-Induced Microglia Activation (*In vivo*)	4	Chronic Binge Exposure (4 Days) in Adult Male Sprague Dawley Rats	5 g/kg, ~300 mg/dl	Hippocampus	↑Proliferation of microglia and astrocytes (28 & 56 days into abstinence)	N/A	Nixon et al., 2008
	5	Chronic Binge (4 Days) in Adult Male Sprague Dawley Rats	5 g/kg, ~300 mg/dl	Hippocampus	↑CD11b, ED1, Proliferation of microglia, TNF-α, & IL-1β	↑ Neuronal apoptosis	Zhao et al., 2013
	6	Chronic Binge Exposure (4 Days) in Adolescent Male Sprague Dawley Rats	5 g/kg, 300-400 mg/dl	Hippocampus	↑IBA-1 & Proliferation of microglia	N/A	McClain et al., 2011
TLR-4 & NFκB	7	Chronic Intermittent Exposure (5 Days) in Postnatal (PND 2-6) Sprague Dawley Rats	2.5 g/kg, 150-200 mg/dl	Hypothalamus	↑IBA-1, activated microglia morphology, TNF-α, MCP1, TLR4, & CSFR1 ↓POMC & spine density *In microglia ↑ IBA-1, MOR, TLR-4, p38MAPK, p-JNK, p-AKT, NFκB	↑ POMC neuronal apoptosis	Shrivastava et al., 2017
	8	*In vitro* – Chronic Exposure (24 or 48 Hour)	50 mM	Primary Rat and Mouse Mixed[#] Culture	↑Microglial activation and phagocytosis, CD11b, TNF-α, IL-1β, Nitrite, & MYD88-dependent & MYD88-independent signaling molecules	↑Neuronal apoptosis	Fernandez-Lizarbe et al., 2009
	9	Human Postmortem Brains (Alcoholic vs. Moderate Drinking Controls) Chronic Binge Exposure in Adolescent (PND 25-55) Male Sprague Dawley Rats	N/A PND 55: ~165 mg/dl	OFC OFC	↑RAGE & Correlation of TLR-4, HMGB1, and RAGE with onset age of drinking ↑RAGE, TLR-4, HMGB1, TNFα, MCP-1, NOX2, COX2 & MYD88 at PND 80	N/A	Vetreno et al., 2014

ROS	10	*In vitro* – Chronic Exposure (2 or 4 days)	25, 50, 100 mM	Primary MBH and Microglia Culture	The effect thanol-activated microglia media on MBH culture neurons: ↑O_2^-, ROS, Nitrite ↓GSH, GHS-Px SOD, Catalase	↑MBH neuronal apoptosis	Boyadjieva & Sarkar, 2013
↑, Increase in gene or protein expression; ↓, Decrease in gene or protein expression; PND, Postnatal day; MBH, Mediobasal hypothalamus; N/A, Not applicable							

1.6.3 COMMUNICATION BETWEEN MICROGLIA AND NEURONS

1.6.3.1 RESPONSE TO HYPERGLUTAMATERGIC STATE

Bidirectional communication between neurons and microglia has been shown in rats following a seizure. In rats, kainic acid-induced seizures produce significant increases in global glutamate release, which activates NMDA receptors, produces a calcium influx, ATP release from neurons, and binding of ATP to microglial P2Y12 receptors. This process is a vital component in the response to seizures; animals without P2Y12 experience worse seizure behaviors and have a shorter latency to seizure onset (Eyo et al., 2014). Another component in the response to glutamate release involves metabotropic glutamate receptor 5 (mGluR5) as mGluR5 has been shown to mediate neuroprotection of neurons (Bao et al., 2001). Byrnes et al. demonstrated *in vitro* that mGluR5 stimulation, via mGluR5 agonist CHPG inhibited microglial activation and reduced microglial proliferation, TNF-α, NO, and ROS production, as well as microglial-induced neurotoxicity in response to LPS. This protection was nullified in mGluR5 knockout mice or by mGluR5 antagonism (Byrnes et al., 2010). Furthermore, mGluR5 can also have anti-apoptotic effects on neurons (Movsesyan et al., 2004; Byrnes et al., 2010). This suggests that following a CNS injury such as focal ischemia or seizure, which augments glutamate release, neurons can signal to microglia to protect the surrounding neurons from apoptosis via activation of microglial mGluR5 or P2Y12.

1.6.3.2 REGULATION OF SYNAPTIC PRUNING

Another example of communication involves the complement system. Astrocytes can release TGF-β and stimulate retinal ganglion cells to produce C1q. C1q is a complement protein and first step for synaptic pruning and phagocytosis of weak synapses. The binding of C1q and C3 to the axons of weak synapses, which is recognized by the complement-3 receptor on microglia, tells the microglia to prune them (Bialas & Stevens, 2013). Thus, astrocytes, retinal ganglion cells, and microglia cooperate with each other to regulate synaptic pruning. Synaptic pruning by microglia can also be regulated by synaptic activity. Schafer et al. found that retinal ganglion cells treated with tetrodotoxin, a potent neurotoxin that inhibits the firing of action potentials, were pruned more than vehicle treated cells because the tetrodotoxin-treated cells were less active (Schafer et al., 2012). Alternatively, exosomes can serve as a form of communication between neurons and microglia. Exosomes are small membrane vesicles of endosomal origin with proteins, lipids, and nucleic acids from the cytoplasm of the cells they originate from. An *in vitro* experiment demonstrated that exosomes produced from depolarized neurons promoted an upregulation of C3 and synaptic pruning of neurons in co-culture. Blockade of C3 protected neurons from synaptic pruning (Bahrini et al., 2015).

1.6.3.3 REGULATION OF NEURAL PRECURSORS AND NEUROGENESIS

Communication with neurons during development is also critical for proper neurogenesis and regulation of neural precursors. First, 95% of microglia found in the proliferative zones of the cortex in rats, macaques, and humans were activated as demonstrated by enlarged soma, fewer and thicker processes, and processes extended

towards surrounding cells, as well as co-labeling of activation markers CD14, F4/80, CD11b, MHC class II, and iNOS. Interestingly, in the subventricular zone of macaques, they also found that there was a time dependent increase in the amount of microglia contacting or engulfing Tbr2+ and Pax6+ neural precursor cells. Tbr2+ contact with microglia increased from only 2.1% of Tbr2+ cells at E50 to 3.5% at E65, to 16.5% at E80, and 34.7% at E100, suggesting that microglia regulate the pool of neural precursor cells. Reduction of microglial activation through administration of minocycline or Dox, or depletion of microglia through clodronate treatment increased the number of neural precursor cells in prenatal brains, suggesting that without microglia, neural precursor cells will proliferate and survive without regulation (Cunningham et al., 2013). These data suggest that microglia play an important role in regulating neural precursor cells and, while a mechanism for communication between microglia and these cells has not been discovered, it is likely that these cells are communicating with each other.

1.6.3.4 AGING AND ITS EFFECT ON COMMUNICATION

Communication between neurons and microglia can also change over time. Aging affects microglia in numerous ways, one of which is called a dystrophic phenotype. This phenotype is a morphological change that is different from the response to an acute challenge; these are permanent changes that result from cumulative activation over time. These changes can result in the loss of function, dysfunction, or hyperactivation (Michell-Robinson et al., 2015). For example, aged microglia produce higher levels of proinflammatory cytokines including TNF-α, IL1-β, and IL-6 and microglial activation markers CD11b, MHC class II, and pathogen-associated molecular pattern receptors (Williams et al., 1992; Perry et al., 1993). Others show a diminished ability to respond to

injury or internalize amyloid-β42 in Alzheimer's disease (Streit et al., 2004; Njie et al., 2012; Hefendehl et al., 2014). Unresponsive or hyperactivated microglia would not be able to properly respond to surrounding neurons and could be detrimental, thus emphasizing the importance of communication between microglia and neurons. Another interesting form of communication I am interested in researching is between β-endorphin neurons and microglia involving MOR and DOR.

1.6.4 EFFECT MICROGLIAL MOR AND DOR RECEPTOR ACTIVATION IN INFLAMAMTION

1.6.4.1 MOR AND PROINFLAMMATION

As mentioned above, β-endorphin can bind to MOR and DOR and these receptors can be found on microglia (Turchan-Cholewo et al., 2008). Knowing this what role do these opioid receptors play in microglial activation? Recent evidence suggests that MOR on microglia participate in neuroinflammation. Content et al. chronically exposed mice to ethanol and found decreased levels of immature DCX neurons in the subgranular zone and an increased immune response, as demonstrated by an increase in IBA-1 staining in the granule cell layer. Interestingly, this effect of ethanol was attenuated in MOR knockout mice, leading the authors to suggest that MOR directly contributes to ethanol-induced activation of microglia and loss of DCX neurons (Content et al., 2014). Merighi et al. went into further detail establishing a potential mechanism for MOR in microglial-induced neuroinflammation. In a primary microglia culture, Merighi et al. found that LPS augmented phosphorylation of PKCε and its translocation to the membrane. LPS also increased the phosphorylation of downstream signaling molecules ERK1/2 and Akt. This signaling mechanism was correlated with increases in cytokine production from primary

microglia, including Il-1β, IL-6, iNOS, and TNF-α. Intriguingly, MOR agonists morphine or [D-Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO) produced synergistic increases with LPS co-administration to the above signaling mechanism. Additionally, inhibition of MOR signaling through siRNA or antagonist treatment blocked the increases observed with LPS co-administration. However, MOR agonist treatment alone was not sufficient to induce proinflammatory cytokine production alone, it requires LPS co-administration, suggesting activation of TLR-4 is necessary (Merighi et al., 2013). These data suggest that MOR activation leads to neuroinflammatory response, which may require TLR-4 activation.

1.6.4.2 DOR AND ANTI-INFLAMMATION

DOR activation appears to play a different role in microglial activation. First, DOR activation in the brain following ischemia was found to be neuroprotective against hypoxic injury. More specifically, DOR administration reduced the number of cells positive for cleaved caspase-3, increased antioxidant enzymes SOD and GSH-px, and decreased malondialdehyde (MDA, a free radical product) and NO production relative to ischemia/reperfusion stress-exposed animals (Yang et al., 2009). In another model of ischemia, Tian et al. found that DOR administration reduced ischemia-induced microglial activation, evidenced by reduced Cd11b expression on microglia, and naltrindole (a DOR antagonist) increased CD11b expression on microglia (Tian et al., 2013). One potential mechanism for DOR's neuroprotective action can be found studying astrocytes. Akhter et al., exposed primary astrocytes to TNF-α to simulate glaucomatous neurodegeneration via increased matrix metalloproteinase-2 (MMP-2) secretion and activity, phosphorylated-p38 MAPK secretion, and NF-κB expression. This upregulation was

blocked by an exogenous DOR ligand SNC-121 (Akhter et al., 2013). Taken together, these data suggest that DOR activation plays a vital role in neuroprotection, possibly through inhibition of p-38 MAPK and NF-κB.

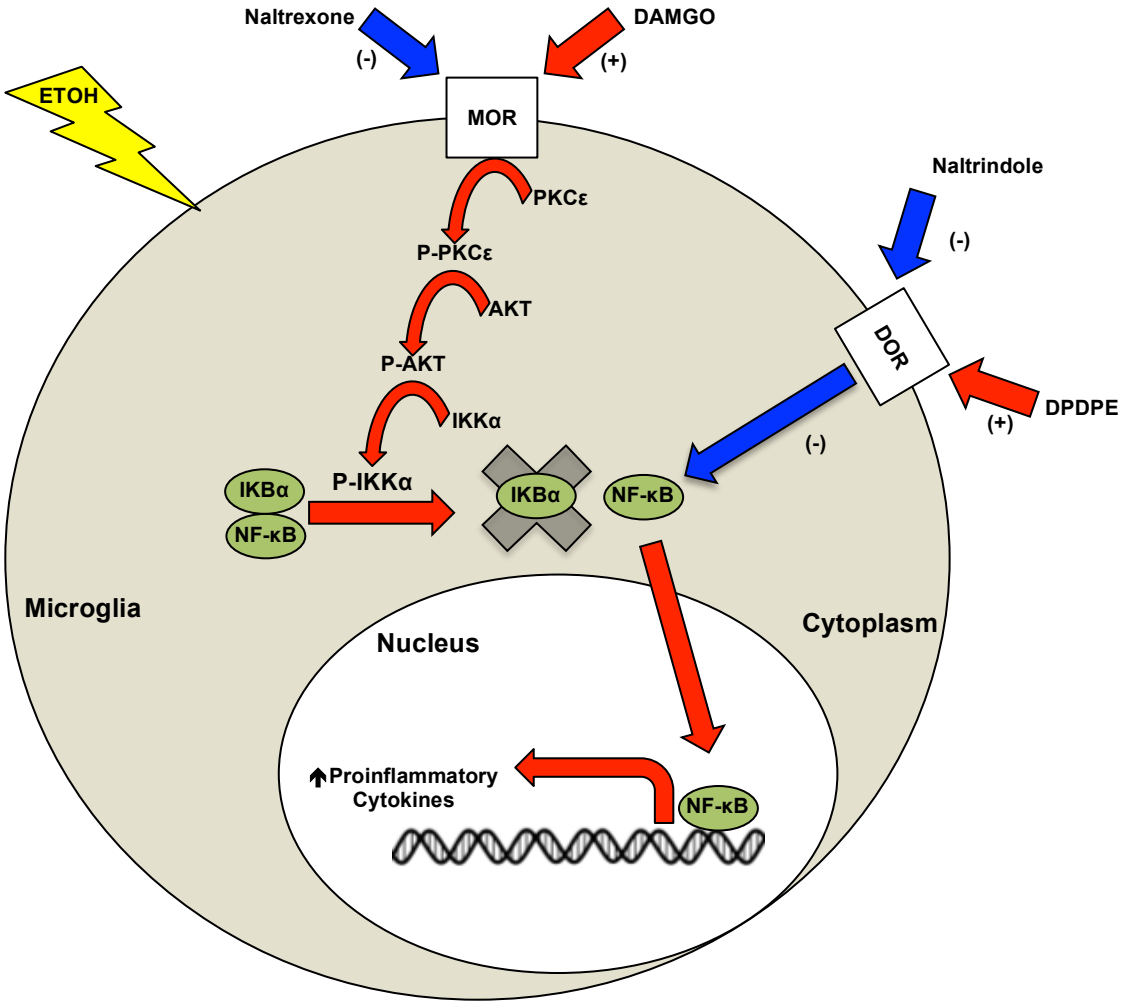


FIGURE 1. POTENTIAL MECHANISM FOR THE EFFECT OF MICROGLIAL MOR AND DOR RECEPTOR ACTIVATION IN INFLAMAMTION

Above is an illustration depicting a potential mechanism through which MOR and DOR receptors regulate microglia in an ethanol-induced inflammatory environment adopted and modified from Chandrasekar et al., 2003, Akhter et al., 2013, and Merighi et al., 2013. MOR agonist DAMGO activates MOR and leads to a signaling cascade involving phosphorylation of PKCε, AKT, IKKα, and IKBα. Phosphorylation of IKBα degrades it and releases NF-κB, which travels to the nucleus and upregulates proinflammatory cytokine transcription in the presence of a TLR-4 activator such as ethanol. DOR agonist

DPDPE activates DOR and suppresses NF-κB to prevent proinflammatory cytokine transcription. Naltrexone and Naltrindole are MOR and DOR antagonists, respectively.

1.6.4.3 EFFECT OF CHRONIC ETHANOL AND OPIOID ANTAGONISM ON MOR AND DOR EXPRESSION

Findings from Dr. Sarkar's lab illustrate that MOR and DOR can be differentially regulated and expressed depending on antagonist treatment. First, Boyadjieva et al. discovered that acute naltrexone treatment dose-dependently inhibited NK cell activity *in vitro* (Boyadjieva et al,. 2001). Then in 2004, they found that chronic naltrexone administration, *in vitro*, increased NK cell activity. This change from inhibition of NK cell activity towards activation was due to changes in MOR and DOR receptor levels. Precisely, they found that chronic naltrexone administration depleted MOR and increased DOR on the surface of NK cells (Boyadjieva et al., 2004). Another study (*in vivo* and *in vitro*) found that chronic treatment of MOR or DOR antagonists increased DOR or MOR monomer and homodimer proteins on NK cells, respectively. Additionally, chronic ethanol exposure induced the heterodimerization of MOR and DOR, which reduced the responsiveness of NK cells to opioids (Sarkar et al., 2012). Moreover, these data suggest that chronic ethanol exposure can alter the function of opioid receptors, as well as opioid antagonists.

Since, postnatal ethanol exposure diminishes the number of β-endorphin neurons in the arcuate nucleus it is likely that communication between β-endorphin neurons and microglia will be altered. Therefore, I will investigate the role of MOR and DOR in ethanol-induced activation of microglia to induce apoptosis of β-endorphin neurons.

1.7 EXOSOMES

1.7.1 OVERVIEW OF EXOSOMES

The secretion of extracellular vesicles has been known for some time and it was first reported in 1946 with extracellular vesicles defined as pro-coagulant, platelet-derived particles (Chargaff & West, 1846; Paolicelli et al., 2018), However, the term "exosomes" was not coined until Johnstone et al. demonstrated that these small extracellular vesicles were derived from an endosomal origin (Johnstone et al., 1987).

Exosomes are small vesicles (30-150 nm in diameter) that form from the inward budding of the limiting membrane of endosomes. This process is thought to be driven by either the endosomal-sorting complex required for transport (ESCRT) or neutral sphingomyelinase 2 (nSMase2). This inward budding results in exosomes containing cargo from the cytoplasm as well as the cell membrane. Following inward budding, exosomes form within multivesicular bodies and are transported to the cell membrane to be released or degraded in lysosomes with the aid of the RAB 7, 11, 27, and 35. They dock at the cell membrane by binding their soluble N-ethylmaleimide-sensitive fusion attachment protein (SNAP) receptors (SNAREs) to the cell membrane SNAPs, resulting in the fusion of both organelles and secretion of exosomes into the extracellular space (Colombo et al., 2014).

The composition of exosomes are heterogeneous and can vary based on the cell origin, however, there are some molecular components that are commonly found in most exosomes. Since exosomes are derived from endosomes, their lipid bilayer is primarily composed of sphingomyelin, phosphatidylserine, cholesterol, and ceramide (Llorente et al., 2013; Colombo et al., 2014). Furthermore, they are composed of numerous proteins, including membrane proteins called tetraspanins (CD63 and CD81), MHC class I and II molecules, lysosome-associated membrane protein (LAMP), and cell adhesion

molecules, as well as cytoplasmic proteins such as ESCRT proteins ALG2-interacting protein X (ALIX), tumor susceptibility gene 101 protein (TSG101), cytoskeleton proteins, and other cytosolic proteins. Genetic information (mRNAs, miRNAs, and other noncoding RNAs) can also be found in exosomes (Colombo et al., 2014). Examples of cell-specific molecules are CD13 and CD11b for microglia, glial fibrillary acidic protein (GFAP) for astrocytes, and ATPase α3 for neurons (Potolicchio et al., 2005; Coleman et al., 2017).

Exosomes can be taken up by neighboring cells through receptor-mediated endocytosis, phagocytosis, macropinocytosis, clathrin-mediated endocytosis, clathrin-independent endocytosis, or membrane fusion (Barres et al., 2004; Barres et al., 2010; Montecalvo et al., 2012; Fitzner et al., 2011; Svensson et al., 2013; Abrami et al., 2013; Gupta et al., 2004; Mulcahy et al., 2014). Once exosomes enter their target cell their cargo is either degraded by lysosomes or released into the cytosol to affect the cell's homeostasis.

1.7.2 EXOSOMES ROLE IN CNS COMMUNICATION

1.7.2.1 EFFECT OF EXTRACELLULAR VESICLES FROM NEURONS AND ASTROCYTES ON MICROGLIA

Exosomes were first shown to be released by neurons and astrocytes in primary cortical cultures. Those exosomes contained GluR2/3 and their release was regulated by depolarization (Fauré et al., 2006). Another study found that cortical neuron exosomes were only endocytosed by other neurons, suggesting that some neuronal exosomes may express specific markers for communication between specific cells (Chivet et al., 2014). Neurons and microglia can also communicate with each other via extracellular vesicles.

An example of this was demonstrated by Antonucci et al. where microglia-derived microvesicles stimulated presynaptic terminals of cultured hippocampal and visual cortex neurons to release glutamate in a dose-dependent manner. This effect occurred because microglia-derived microvesicles enhanced sphingolipid metabolism and produced greater amounts of ceramide and sphingosine, which induced the release of glutamate from secretory vesicles docked at the presynaptic terminal (Antonucci et al., 2012). Another example of neurons communicating with microglia through extracellular vesicles can be found in Bahrini et al. In this study they collected exosomes from PC-12 cells following exposure to 25mM KCl depolarization for 3 hours. These exosomes were then pre-incubated with MG6 microglia cells for 16 hours and synaptic pruning of PC-12 cells was measured. MG6 cells, which were pre-incubated with depolarized neuronal exosomes, enacted greater synaptic pruning of PC-12 co-cultured cells relative to non-exposed MG6 microglia cells. This acceleration was aided by greater expression of complement component 3 by the MG6 cells (Bahrini et al., 2015). Extracellular ATP has also been shown to augment the release of extracellular vesicles from microglia that contain proteins involved in autophagy, energy metabolism, cell adhesion, phagocytosis, endocytosis, and apoptosis (Drago et al., 2017). Lastly, neurons can communicate with microglia through release of serotonin. Binding of serotonin to microglial serotonin receptors induces phospholipase C-mediated influx of calcium and stimulates exosome release (Glebov et al., 2015). The functions of those exosomes were not determined in this study but those exosomes could regulate activity of the neurons secreting serotonin. All of these experiments demonstrated how neuronal signals activate microglia to release

extracellular vesicles. The next section will explore how exosomes can activate microglia to promote apoptosis of neurons.

1.7.2.2 MICROGLIAL EXOSOME RESPONSE TO INFLAMMATORY CONDITIONS

The ability of exosomes to serve as a form of communication between neurons can also induce apoptosis in neurons. In a murine model of Alzheimer's disease the amyloid precursor protein (APP), c-terminal fragments of APP, and numerous proteases were found packaged into exosomes and their secretion could be augmented by inhibition of gamma-secretase (Sharples et al., 2008). Furthermore, within these exosomes APP can be cleaved by β-secretase to generate amyloid β-protein (Aβ) (Rajendran et al., 2006). Microglia are known to eliminate these plaque buildups through phagocytosis, however, reactive microglia can also produce extracellular vesicles containing the neurotoxic Aβ and release them to neighboring cells (Joshi et al., 2014). Transmission of these Aβ molecules can be toxic and induce apoptosis in astrocytes (Wang et al., 2012). Inhibition of exosome production with GW4869 or deletion of nSMase2 in culture reduced Aβ load, tau phosphorylation, and glial activation, improved cognition in a fear condition task, and ameliorated glial apoptosis (Wang et al., 2012; Dinkins et al., 2014; Dinkins et al., 2016). In Parkinson's disease (PD) exosome release from microglia is stimulated by alpha-synuclein, which is found to aggregate in neurons, induce apoptosis via mitochondrial mechanisms, and activate microglia (Chang et al, 2013). Furthermore, cerebrospinal fluid exosomes from PD patients were found to have elevated levels of alpha-synuclein and those exosomes could induce oligomerization of alpha-synuclein in reporter cells (Stuendl et al., 2016). Oligomerization of alpha-synuclein represents a major constituent

of Lewy Bodies and produce neurotoxicity. Additionally, administration of LPS can activate microglia to release extracellular vesicles containing IL-1β and microRNA-155 and promote neuroinflammation (Jablonski et al., 2016; Kumar et al., 2017). Astrocyte-derived ATP can also induce microglial extracellular vesicle release in a co-culture model. Extracellular vesicle release occurs following the binding of ATP to microglial P2X7. In addition, these microglial extracellular vesicles contained precursor of interleukin-1 beta (pro-IL1-β), IL-1β, and caspase-1, which can promote neuroinflammation. Interestingly, caspase-1 is required to cleave pro-IL-1β and produce IL-1β, thus IL-1β levels could be enriched within the extracellular vesicles after they leave microglia (Bianco et al., 2005). These data suggest that microglia can use exosomes to communicate with neurons and can, in a proinflammatory or neurodegenerative environment, contribute to the apoptosis of neurons through exosomes. In this proposal I will determine the role exosomes play in ethanol-induced apoptosis of BEP neurons by microglia.

1.8 COMPLEMENT SYSTEM

1.8.1 OVERVIEW OF FUNCTION AND SIGNALING MECHANISMS

The complement system is a critical regulator of the immune system, peripherally and centrally, regulating phagocytosis of apoptotic cells, synaptic pruning, and regulation of inflammation (Stevens et al., 2007; Griffiths et al., 2009; Bohlson et al., 2014). These processes are regulated through 3 distinct pathways (classical, alternative, and lectin). The classical pathway begins with the binding of C1q to apoptotic cells via recognition of apoptotic cell associated molecular patterns (ACAMPs) such as phosphatidylserine or damage associated molecular patterns (DAMPs) like HMGB1. These two molecules can

be described as “eat me” signals, which direct immune cells to phagocytose these apoptotic cells. C1q can also recognize IgM or IgG bound to antigens or pathogen associated molecular patterns such as LPS. C1q binds with the aforementioned molecules and forms an activated C1 complex comprised of 1 C1q molecule, 2 C1r molecules, and 2 C1s molecules. This activation leads to the generation of the C1s esterase which cleaves C4 and C2 complement proteins. The cleavage products of C4 and C2 (C4b and C2a) bind together and form the C3 convertase. C3 convertase then cleaves C3 into C3a and C3b. C3b binds to the C3 convertase and generates the C5 convertase (C4b2a3b). The C5 convertase continues to cleave C5 and promotes the formation of a transmembrane pore called the membrane attack complex (MAC; C5b-C9) for opsinization and cell lysis (Rus et al., 2005). Alternatively, the binding of mannose-binding lectin (MBL) to bacterial surface carbohydrates activates the lectin pathway. Once activated MBL-associated serine proteases (MASP) 1, 2, and 3 bind to the surface and activate the MASP esterase. MASP esterase then cleaves C4 and C2 and generates C3 convertase (C4bC2a) similarly to the classical pathway (Petersen et al, 2000; Dahl et al. 2001). The rest of the pathway proceeds similarly to the classical pathway leading to the generation of MACs. Finally, the alternative pathway is activated by the alteration of C3 to C3(H_2O), which can occur autonomously. Factor B associates with C3(H_2O) leading to a change in confirmation for Factor B. This confirmation change allows serum protease Factor D to cleave Factor B to generate Ba and Bb. The Bb molecule stays bound to C3 and can cleave an additional C3 molecule producing C3bBb, an alternative C3 convertase. The C3 convertase is considered unstable and the binding of properdin

can stabilize the protein-protein interactions (Thurman & Holers, 2006). C3 convertase can then cleave more C3 and induce the formation of MACs.

1.8.2 COMPLEMENT SYSTEM IN THE CNS

In the central nervous system, microglia, astrocytes, and neuronal cells to a lesser extent produce the complement component proteins. Furthermore, microglia have both C3 receptors (CD11b) and C5 receptors, which are critical for stimulating cytokine release, phagocytosis of complement-bound compounds, and chemotaxis (Veerhuis et al., 1999; Stephan et al., 2012; Fonseca et al., 2017). During development, under normal physiological conditions, complement components promote neurogenesis and cell migration. Inhibition of serping1, a C1-inhibitor, impairs neuronal stem cell differentiation and migration, while a C5a receptor agonist rescues this deficit (Anna et al., 2017). Additionally, ischemia-induced neurogenesis was decreased in the hippocampus of C3 knockout mice (Rahpeymai et al., 2006). Moreover, the complement system regulates synaptic pruning in a TGF-β dependent manner. TGF-β release from astrocytes primes neurons to release C1q, which bind to weak synapses and interact with microglia complement receptors and promote phagocytosis (Bialas & Stevens, 2013). However, unlike the periphery where cells express complement inhibitors such as CD59 and Factor H on the surface, neurons do not express a majority of complement inhibitors (Cahoy et al., 2008; Stephan et al., 2012;). This suggests that neurons are more prone to complement dysregulation and disease. Evidence for this can be found in Alzheimer's disease where increased C1q is associated with increased synaptic pruning prior to amyloid beta plaques formation (Hong et al., 2016). Similarly there is an increase in C1q and C3 expression in the hippocampus of patients with multiple sclerosis resulting in a

marked decrease in synaptic density. This also occurs in mouse retina during the early stages of glaucoma, prior to the detection of neuronal loss (Steele et al., 2006). Finally, even normal aging promotes complement proteins such as C1q in the hippocampus and reduces synaptic density (Stephan et al., 2017).

1.8.3 EFFECT OF ETHANOL EXPOSURE ON COMPLEMENT SIGNALING

While dysregulation of the complement system can be found in the early stages of neurological diseases, what effect does ethanol exposure have on the complement system? Chronic ethanol exposure in mice promoted complement component proteins and downregulated complement inhibitors (Factor-H, MASP-2, and Factor-D) in the liver (Bykov et al., 2007). Furthermore, chronic ethanol exposure increased C1q and C3 deposition on apoptotic Kupffer cells and TNF-α and IL-6 production, suggesting a role in the pathogenesis of ethanol-induced liver injury (Cohen et al., 2009; Roychowdhury et al., 2010). Moreover, chronic ethanol exposure also affects adipose tissue by upregulating cytochrome P450 expression and activating C1q and Bid-mediated apoptosis to produce adipose tissue inflammation (Sebastian et al., 2011). However, more work needs to be done to assess the effects of chronic ethanol exposure on the complement system and neuronal homeostasis.

1.8.4 INTRACELLULAR COMPLEMENT SIGNALING

Complement proteins also play a critical role in the regulation of cell survival through intracellular signaling. For example, C1q can bind to mitochondria through the globular C1q receptor (gC1qR) to drive ROS production and neuronal apoptosis following hypoxia-mediated damage or cortical ischemia (Dedio et al., 1998; Ten et al., 2010). Another study found that human cervical squamous carcinoma cells downregulate

gC1qR expression to prevent gC1qR-induced apoptosis of those cancer cells via a mitochondrial driven pathway (Chen et al., 2014). Furthermore, the C1 complex can enter a late apoptotic cell and bind to its nucleoli resulting in the activation of the proteases C1r/C1s. Activation of this complex results in cleavage of numerous nuclear proteins including nucleolin and nucleophosmin-1 (Cai et al., 2015). Complement C3 is also an important intracellular signaling molecule, especially in T cells. Liszewiski et al. found that T cells contain stores of endosomal and lysosomal C3, along with cathepsin L that can cleave C3 into C3a and C3b. Tonic generation of C3a was required to maintain T cell homeostasis and survival while alterations in C3a resulted in an induced autocrine proinflammatory cytokine production that could only be rescued by cathepsin L inhibition (Liszewski et al., 2013). Tam et al. found that internalization of C3, whether it was attached to a virus or coated beads, activated NFκB, interferon regulator factors (3, 5, and 7), and AP-1. This led to an augmented production of cytokines IL-6, TNF-α, CCLA, IL-1β, and IFN-β. This signaling cascade was mitochondrial antiviral- and TRAF6-dependent (Tam et al., 2014). These data suggest that complement proteins are critical to the survival of immune and non-immune cells and dysregulation of intracellular complement signaling can lead to apoptosis. I will investigate the role intracellular complement signaling may play in ethanol-induced BEP neuronal apoptosis.

HYPOTHESIS

Ethanol-induced killing of β-endorphin neurons is caused by mu-opioid receptor activated proinflammation, secretion of exosomes containing pro-apoptotic and proinflammatory factors, complement component signaling, and reduced delta-opioid receptor anti-inflammation.

AIMS OF THIS STUDY

Aim 1: To determine the role MOR and DOR play in ethanol activation of microglia to promote apoptosis of β-endorphin neurons.

Aim 2: To assess the role exosomes have in ethanol's neurotoxic effect on β-endorphin neurons.

Aim 3: To establish whether the complement system mediates ethanol's neurotoxic effect on β-endorphin neurons.

CHAPTER 2

2.1 INTRODUCTION

Microglia are one of the innate immune cells of the brain and represent about 10% of the total brain cell population (Alliot et al., 1999). Microglia behave similarly to macrophages and, under normal physiological conditions, maintain neuronal homeostasis (Kettenmann et al., 2011). Microglial activation falls on a spectrum but exhibits two distinct phenotypes labeled M1 and M2. The M2 phenotype has a distinct morphology with long, thin processes and small, elliptical soma. These ramified microglia maintain homeostasis through the secretion of neurotropic factors and anti-inflammatory cytokines (Tam et al., 2016). However, when exposed to a toxicant or insult, microglia shift their phenotype to an M1 phenotype. This M1 phenotype is typically associated with an increased soma size, thicker and shorter processes, increased release of proinflammatory factors such as TNF-α, IL-1β, IL-6, and MCP1, as well as upregulated TLR4 and IBA-1 (Chhor et al., 2013; Chastain & Sarkar, 2014). Activation of microglia to the M1 phenotype is particularly detrimental during the fetal developmental time period and may lead to neurotoxicity and developmental disorders (Bilbo & Schwarz, 2012).

Ethanol exposure is also known to activate microglia to induce neurotoxicity and apoptosis in developing neurons. *In vitro* experiments have demonstrated this in primary microglial culture where ethanol exposure activated microglia and increased proinflammatory cytokine (TNF-α, IL-6, and MIP-2) release. Furthermore, conditioned media from these microglia induced apoptosis of immature neurons in a primary hypothalamic culture via upregulation of ROS and decreased expression of BDNF, cAMP, and antioxidant enzymes (Boyadjieva et al., 2010; Boyadjieva & Sarkar, 2013).

Animal studies have also demonstrated this link between ethanol and microglia. Prenatal or postnatal ethanol exposure has been shown to induce microglial activation, neuronal apoptosis, loss of synaptic proteins, and phagocytosis of degenerating neurons (Saito et al., 2012; Saito et al., 2016; Pascual et al., 2017; Wang et al., 2018). Importantly, ethanol-induced microglial activation and neuronal apoptosis were blocked when minocycline, a non-selective microglial inhibitor, was administered prior to ethanol exposure (Wang et al., 2018). Notably, these experiments model a third trimester equivalent paradigm in humans, where alcohol consumption has been linked to numerous detrimental effects on the developing brain.

Alcohol exposure during pregnancy may cause FASDs. Many FASDs patients exhibit lifelong stress, cognitive, growth, and immune abnormalities (Bertrand et al., 2004; Zhang et al., 2005; CDC, 2008). Examples of stress abnormalities have been demonstrated by augmented responses to stress hormones including ACTH and corticosterone (Lee et al., 1990; Lee et al., 2016). Animal studies have demonstrated that gestational ethanol exposure alters the function of the HPA axis, which may, in part, be due to the reduction in function and number of stress regulatory β-endorphin neurons in the arcuate nucleus of the hypothalamus (Weinberg et al., 2008; Logan et al., 2015).

Interactions between microglia and neurons are vital to maintain homeostasis, suggesting some form of communication occurs. Examples of this communication can be found in response to ischemia, seizure, inflammation and neurodegenerative disease, and regulation of synaptic pruning and neurogenesis (Movsesyan et al., 2004; Sterit et al., 2004; Byrnes et al., 2010; Bilias & Stevens, 2013; Cunningham et al., 2013; Eyo et al., 2014). Furthermore, microglia express purinergic, glutamatergic, GABAergic,

cholinergic, dopaminergic, and opioidergic receptors the can respond to neurotransmitters (Kettenmann et al., 2011). Since β-endorphin is an opioid peptide that can bind to and activate MOR and DOR, it is possible that these receptors are involved in communication between β-endorphin neurons and microglia during ethanol-induced neurotoxicity.

Utilizing a third trimester equivalent ethanol-feeding model in rats, I demonstrated that ethanol exposure activates microglia and induces apoptosis of β-endorphin neurons. Then I evaluated the role of MOR and DOR in ethanol-induced activation of microglia. Here I provide evidence that ethanol's neurotoxic actions on β-endorphin neurons are the result of microglial activation and the differential effects of MOR and DOR on microglia. Specifically, MOR promotes neuroinflammatory signaling from microglia but DOR does not.

2.2 MATERIALS AND METHODS

Animal Use

Animal care and treatment were performed in accordance with institutional guidelines and complied with the National Institutes of Health policy and Rutgers Animal Care and Facilities Committee. Adult Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). Adult transgenic mice (C57BL/6J) expressing the enhanced green fluorescent protein (EGFP) in POMC neurons were obtained from Dr. Malcolm Low's laboratory at Oregon Health & Sciences University, Portland. Rats and mice were kept under 12 hour light/12 hour dark conditions, provided water and rodent chow ad libitum, and mated. Neonates were used for postnatal ethanol exposure experiments.

Postnatal Ethanol Exposure Model

Postnatal rat or mouse pups (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 isocaloric control (PF), or they were left in the litter with their mother (AD). Gavage feeding was conducted at 10:00 AM and 12:00 PM from PND 2-6. After feeding, these pups were immediately returned to the litter. Additionally, some animals were treated subcutaneously with minocycline (45μg/kg; one hour prior to the first feeding), or naltrexone (NTX, 10mg/kg; 15 minutes prior to the first feeding), or naltrindole (NTD, 10mg/kg; 15 minutes prior to the first feeding). All of these drugs were purchased from Sigma Aldrich (St. Louis, MO). Two hours after the last feeding on PND 6, some of the pups were transcardially perfused with 4% paraformaldehyde, postfixed overnight, cryoprotected in 30% sucrose, and cut into 30

μm coronal sections for immunohistochemistry. Additional pups were sacrificed and the mediobasal hypothalamus (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 microglia isolation by Optiprep gradient separation method or frozen for measurement of protein or gene measurement.

Immunohistochemistry

Serial coronal sections of the 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, one PF, and one AF brain section. The sections were washed in PBS twice followed by antigen retrieval in citrate buffer (pH 6.2) at 100°C for 10 min. After two washes in PBS-T (0.05% Triton-X) the sections were incubated in blocking buffer (2.5% normal horse serum in PBS-T) at room temperature for 30 min. The sections were subsequently incubated overnight at room temperature with primary antibodies. The primary antibodies for immunohistochemistry were used as follows: goat anti-IBA-1 (1:500; Abcam, Cambridge, MA), rabbit anti-DOR (1:50; Santa Cruz, Dallas, TX), rabbit anti-MOR (1:500; Antibodies Inc., Davis, CA), rabbit anti-GFP (1:2000; Abcam, Cambridge, MA), and rabbit anti-ß-endorphin (1:500, Peninsula Laboratories, San Carlos, CA). After the primary antibody incubation the samples were washed in PBS and then sections were incubated with peroxidase-coupled secondary antibodies (ImmPRESS reagent; Vector Laboratories, Inc., Burlingame, CA) for 3,3′-diaminobenzidine peroxidase (DAB) or Alexa Fluor secondary antibodies (Thermo

Fisher Scientific, Grand Island, NY) for immunofluorescence. For DAB staining, antigen localization was achieved by using the 3,3′-diaminobenzidine peroxidase reaction kit (Vector Laboratories, Burlingame, CA). After DAB staining, sections were dehydrated in ethanol and mounted in Permount (Thermo Fischer Scientific). For immunofluorescence, sections were mounted with DAPI (Vector Laboratories, Burlingame, CA) and sealed with nail polish. To evaluate the immunohistochemical staining intensity, sections from each experimental group were photographed using Nikon-TE 2000 inverted microscope (Nikon Instruments Inc., Melville, NY). Pixel density and cell counting were quantified using ImageJ software (National Institutes of Health, Bethesda, MD). For 3D morphological analysis of POMC and microglial interactions, confocal images (Zeiss LSM 710; Oberkochen, Germany) were created using a 20× objective and stacked at 1 mm/step, resulting in 10 mm images. 3D interaction analysis between microglia and POMC neurons were performed using Imaris 8.2 (Bitplane, Concord, MA).

Golgi-Cox Impregnation

Following decapitation brains were rapidly extracted and placed in double distilled water to remove excess blood. Next, the brains were carefully dissected with two transverse cuts: one rostral to the medial basal hypothalamus and another at the cerebellum. The brains were then placed in 5 mL of Golgi-Cox solution overnight in the dark at room temperature. The Golgi-Cox solution was prepared from 5 parts of 5% Potassium Dichromate, 5 parts of 5% Mercuric Chloride, 4 parts of 5% Potassium Chromate, and 10 parts of double distilled water. All solutions were prepared in double distilled water at least 2 days prior to brain extraction. The next day brains were transferred into 5 mL of fresh Golgi-Cox solution for an additional 2 weeks in the dark at

room temperature. The brains were subsequently transferred to a 30% sucrose solution for 2 days at 4° Celsius. The brains were mounted and then cut into 100mm thick coronal sections at -20° Celsius in a cryostat (Leica Microsystems, Inc. CM1900, Wetzlar, Germany). Sections were mounted onto gelatin-coated slides on a small drop of Solution C (FD NeuroTechnologies Inc. FD Rapid GolgiStain Kit, Columbia, MD) and allowed to dry in the dark for 2 days. The sections were washed 2 times, 4 minutes each, in double distilled water. The sections were then placed in 1 part Solution D, 1 part Solution E, and 2 parts of double distilled water for 10 minutes (FD NeuroTechnologies Inc. FD Rapid GolgiStain Kit, Columbia, MD). The sections were rinsed 2 times, 4 minutes each and then dehydrated in increasing solutions of ethanol (50%, 75%, and 95%) for 4 minutes each. Dehydration continued in absolute ethanol (100%), 4 times, 4 minutes each. The sections were then cleared in xylene, 3 times, 4 minutes each and mounted with Permount.

Spine Density Analysis

Slice analysis was performed using a Zeiss LSM 710 confocal microscope. Images were acquired with the reflected light using a transmitted PMT module. Confocal images were created using 100x oil immersion objective and stacked at 0.5 μm/step. Spines were randomly selected from the MBH for each treatment group (3 sections per animal). Spine density wase manually calculated per 10 μm of dendritic length from 51-57 dendrites. Spines were then classified by morphology according to Risher et al., as filopodia, long thin, thin, stubby, mushroom, and branched (Risher et al., 2014). In addition, the lengths of each dendritic spine were measured (RECONSTRUCT, SynapseWeb) and analyzed.

Microglial Separation and Flow Cytometry

Microglial cells were isolated from the MBH of three combined PND 6 pups (both sexes) using the Optiprep density gradient (Sigma Aldrich, St. Louis, MO) and methods described previously with some minor modifications (Lee and Tansey, 2013). Briefly, MBH tissue samples were isolated and mechanically dissociated using 18-gauge needle followed by a 21-gauge needle in Hank's buffered salt solution (HBSS) media. The cells were strained through a 40 μm strainer to remove myelin. They were then trypsinized (0.5% trypsin) to digest tissue. Adding HBSS + 10% fetal bovine serum media stopped the trypsinization reaction. Next, samples were loaded on an Optiprep column. Optiprep columns were prepared by diluting Optiprep with MOPS (3-(N morpholino) propanesulfonic acid) buffer (0.15 M NaCl, 10 mM MOPS, pH 7.4) in equal quantities. The diluted Optiprep was again diluted in different proportions as 35%, 25%, 20%, and 15% in HBSS media. These solutions were then loaded in a series with the densest solution on the bottom and least dense on top (35%, 25%, 20%, and 15%). Isolated cells were then loaded on top of the columns and then were centrifuged at 1900 rpm for 15 min at 20 °C. The microglia and red blood cells (RBC) were gathered into a pellet at the bottom of the column. The pellet was then taken and incubated with 0.85% ammonium chloride in PBS to lyse RBCs. The remaining purified microglia were washed with PBS 2 times and fixed with 4% paraformaldehyde for 10 min. The cells were then stained for IBA-1 (microglial marker), GFAP (astrocyte marker), and MAP2 (neuronal marker) to determine the purity of microglia. The isolated microglia were >90% pure. These purified microglia were used for protein measurements through flow cytometry analysis. For this, isolated microglia were stained with primary antibodies, rabbit anti-

IBA-1 (1:100; Wako Pure Chemical Industries, USA), rabbit anti-DOR (1:100; Santa Cruz, Billerica, MA), rabbit anti-MOR (1:100; Antibodies Inc, Atlanta, GA), mouse anti-TLR-4 (1:100; Abcam, Cambridge, MA), rabbit anti-P-38 MAPK, IκBα, phosphorylated -Jun N-terminal kinase (P-JNK), and P-AKT (1:100: Cell signaling, Danvers, MA), and mouse anti-NF-κB (1:100, Millipore, Billerica, MA). The cells were labeled with FITC-488 secondary antibody (1:400, Abcam, Cambridge, MA) respective to their primary host and then analyzed by BD Accuri C6 Flow Cytometry. 5000 events per sample were read for all samples and data analysis was completed with C6 Accuri software. Flow cytometric gates were set using forward and side scatter plots and labeled cells were read on the FL-1A (488) channel. The median fluorescent intensity (MFI) values of positively labeled cells were expressed as mean ± SEM of the entire sample, and data is represented as % AD control for all groups to account for variations in fluorescent intensities between batches..

Western blot

MBH tissues were processed for protein extraction followed by quantification of total protein levels via the Bradford Assay (Bio-Rad Laboratories, Hercules, CA). 50 µg of MBH protein were run in 4-20% SDS PAGE and transferred to nitrocellulose membranes at 30 V overnight at 4°C. The membranes were blocked in Odyssey blocking buffer (LI-COR Biotechnology, Lincoln, NE) in PBS at 4°C for 5 hours. The membranes were incubated with primary antibody, anti-TNF-α (1:1000; Abcam, Cambridge, MA), at 4°C overnight. Next, the membranes were washed in PBS with 0.1% Tween-20 and then incubated with corresponding infrared secondary antibody at room temperature for 90 minutes. The membranes were washed and scanned in an Odyssey Infrared Imaging

System (LI-COR Biotechnology, Lincoln, NE). The protein band intensities were determined by Image Studio Lite (LI-COR Biotechnology, Lincoln, NE) and normalized with corresponding β-actin intensity for each sample.

Real-time RT-PCR

Gene expression levels in MBH tissue samples were measured by quantitative real time PCR (SYBR green assay). Total RNA for each MBH sample was extracted using the RNeasy purification kit (Quiagen, Valenica CA) and converted to their first strand complementary DNA (cDNA) using the high capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA). The following primers were used: Forward/Reverse TLR-4 (TGCCTCTCTTGCATCTGGCTGG/CTGTCAGTACCAAGGTTGAGAGCTGG), CSFR1 (GCTCGATGTCCTGCTCTGTGA/CCTGCACTCCATCCATGTCA), MCP1 F/R (GGCCTGTTGTTCACAGTTGCT/TCTCACTTGGTTCTGGTCCAGT), and GAPDH F/R (AGACAGCCGCATCTTCTTGT/CTTGCCGTGGGTAGAGTCAT). PCR was performed at 95°C for 5 minutes, followed by 45 cycles of 95°C for 30 seconds, 62°C for 30 seconds, 72°C for 40 seconds in the Applied Biosystems 7500 Real time PCR system (ABI Carlsbad, CA). Relative quantities of mRNA were calculated by relating the PCR threshold cycle obtained from the tested sample to relative standard curves generated from a serial dilution of cDNA prepared from total cDNA and then quantified as a ratio of GAPDH.

Statistical Analysis

Statistical analyses were conducted using Graph Pad Prism 6.0 software for Mac (Graph Pad Software, Inc., San Diego, CA). Treatment groups were compared by one-way and two-way ANOVAs with Newman-Kuels post-hoc tests. A value of $P<0.05$ was considered significant.

2.3 RESULTS

Using a postnatal ethanol exposure model in rats (PND 2-6; equivalent to the human third trimester) I determined the levels of proinflammatory cytokine protein (TNF-α) and mRNA expression (*MCP-1*, *CSFR1*, and *TLR-4*) in the mediobasal hypothalamus. These factors are known to be upregulated during microglia activation and can promote apoptosis (Beattie, 2014; Chastain & Sarkar, 2014; Zhang et al., 2017). Protein expression for TNF-α and mRNA expression for *TLR*-4 were both found to be elevated in AF-treated rats relative to AD and PF controls (Fig. 2a, d). *MCP1* and *CSFR1* mRNA levels were found to elevated in the mediobasal hypothalamus of AF animals relative to AD controls (Fig. 2b-c). Therefore, postnatal ethanol exposure increased proinflammation production in the MBH.

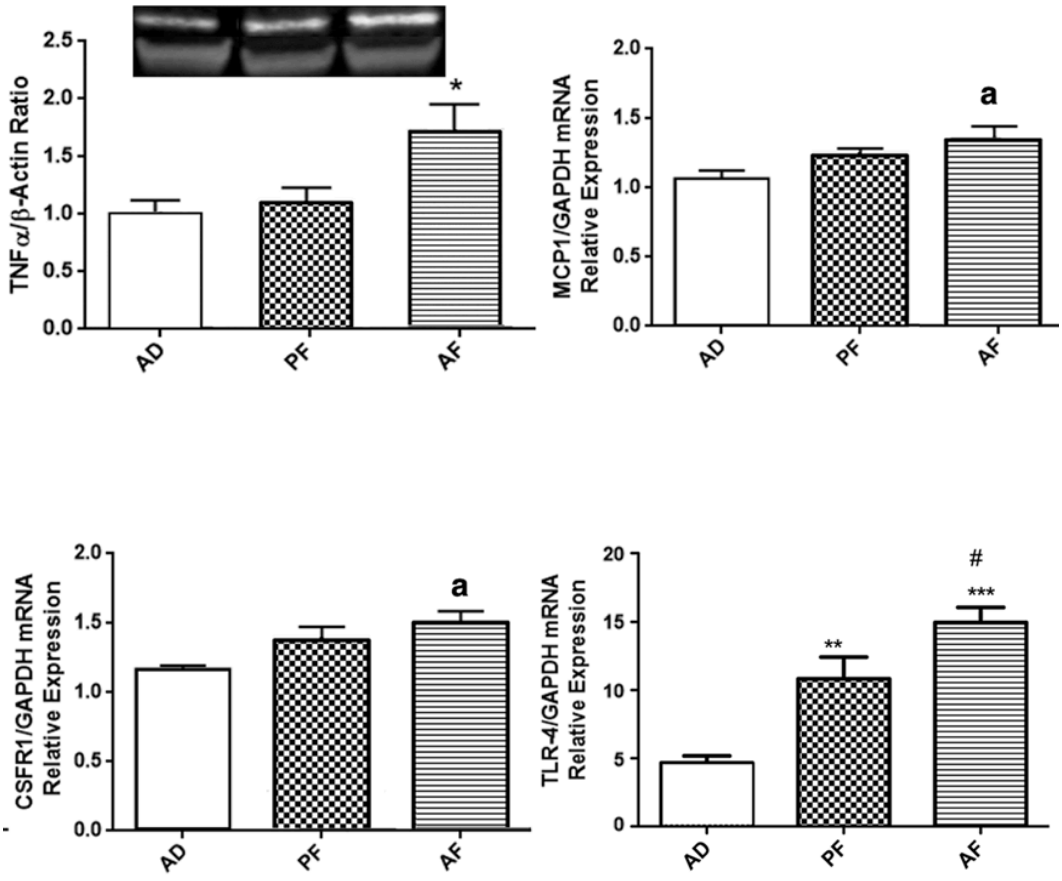


FIGURE 2. POSTNATAL ETHANOL EXPOSURE AUGMENTS PROINFLAMMATORY PRODUCTION

Postnatal ethanol exposure increases the expression of proinflammatory protein and mRNA expression. Histograms above show changes in the protein level of inflammatory cytokine TNF-α (a), mRNA level of cytokine *MCP1* (b), cytokine receptors *CSFR1* (c), and *TLR-4* (d) in the MBH of AF-, PF- and AD-treated rats on PND 6 as determined through Western blot and q-RTPCR, respectively. Data are represented as mean ± SEM (n=5–7). The differences between AD, PF, and AF were compared by one-way analysis of variance (ANOVA) and the Newman-Keuls posttest. *, $p < 0.05$, AF vs PF and AD, **, $p < 0.05$, PF vs AD, ***$p < 0.001$, AF vs PF, a, $p < 0.05$, AF vs AD, , #$p < 0.01$, AF vs PF.

As mentioned earlier, microglia are one of the immune cells of the brain. Activated microglia, in response to ethanol or another insult, can produce proinflammatory cytokines that induce neuronal apoptosis. Therefore, I determined whether fetal ethanol exposure activated microglia by counting cells positive for IBA-1, a microglia specific protein, in the arcuate nucleus of the hypothalamus. The number of IBA-1 positive microglia was increased in the arcuate nucleus of AF-treated ratscompared to PF and AD controls (Fig. 3a, b). Microglia can also be characterized on a spectrum from resting and fully ramified (small soma with longer and thinner processes) to fully amoeboid (completely activated with a very large soma and few, if any processes) microglia. These morphological changes were assessed and counted below. AF animals had significantly more partially and fully amoeboid microglia compared to PF and AD controls (Fig. 3c-e). There was no difference in the number of partially ramified microglia. These data demonstrate that postnatal ethanol exposure increases microglia number and shifts their morphology into an activated phenotype.

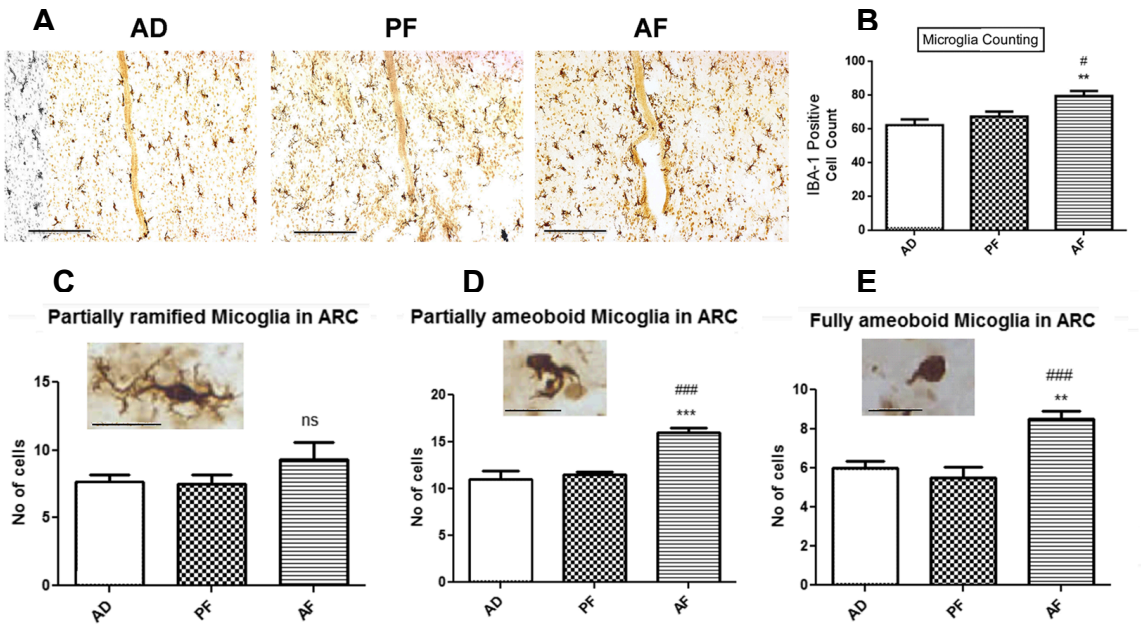


FIGURE 3. POSTNATAL ETHANOL EXPOSURE ACTIVATES MICROGLIA IN THE ACRUCATE NUCLEUS

Postnatal ethanol exposure increases the number of amoeboid and total microglia in the arcuate nucleus. Above are representative images and histograms of IBA-1 positive microglial staining of the arcuate nucleus. Postnatal ethanol exposure increased microglia number (Fig. 1a, b). Scale bars for these images are 200 μm/each. Characterization of IBA-1-stained microglia in the arcuate nucleus based on circularity: partially ramified (c), partially amoeboid (d), and fully amoeboid (e) in AD-, PF-, and AF-treated rat pups on PND 6. Scale bars in these figures are 20 μm/each. Data are represented as mean ± SEM (n=5–7). The differences between AD, PF, and AF were compared by one-way analysis of variance (ANOVA) and the Newman-Keuls posttest. * *, $p<0.01$, ***, $p<0.001$ AF vs AD. #, $p>0.05$, ###, $p<0.001$ AF vs PF

Microglial regulate synaptic pruning, however, in cases of neuroinflammation synaptic pruning can become dysregulated and lead to the loss of dendritic spines. Therefore, I determined whether ethanol-induced microglial activation alters synaptic homeostasis in the hypothalamus. Below are representative images for Golgi staining of dendrites in the hypothalamus (Fig. 4a). I found that postnatal ethanol exposure decreased the length (Fig. 4b) and number of dendrites (Fig. 4c) in the hypothalamus. Furthermore, AF-treated rats had altered dendritic morphology with increased stubby dendrites (Fig. 4d) and decreased mushroom dendrites (Fig. 4h). Stubby and mushroom dendrites represent immature and mature dendrites, respectively. Therefore I combined the data for all immature and mature dendrites and found that there was an overall treatment effect for mature (Fig. 4j) and immature dendrites (Fig. 4k). Taken together, these data suggest that postnatal ethanol exposure alters hypothalamic dendrite number and morphology, resulting in decreased and less mature dendrites.

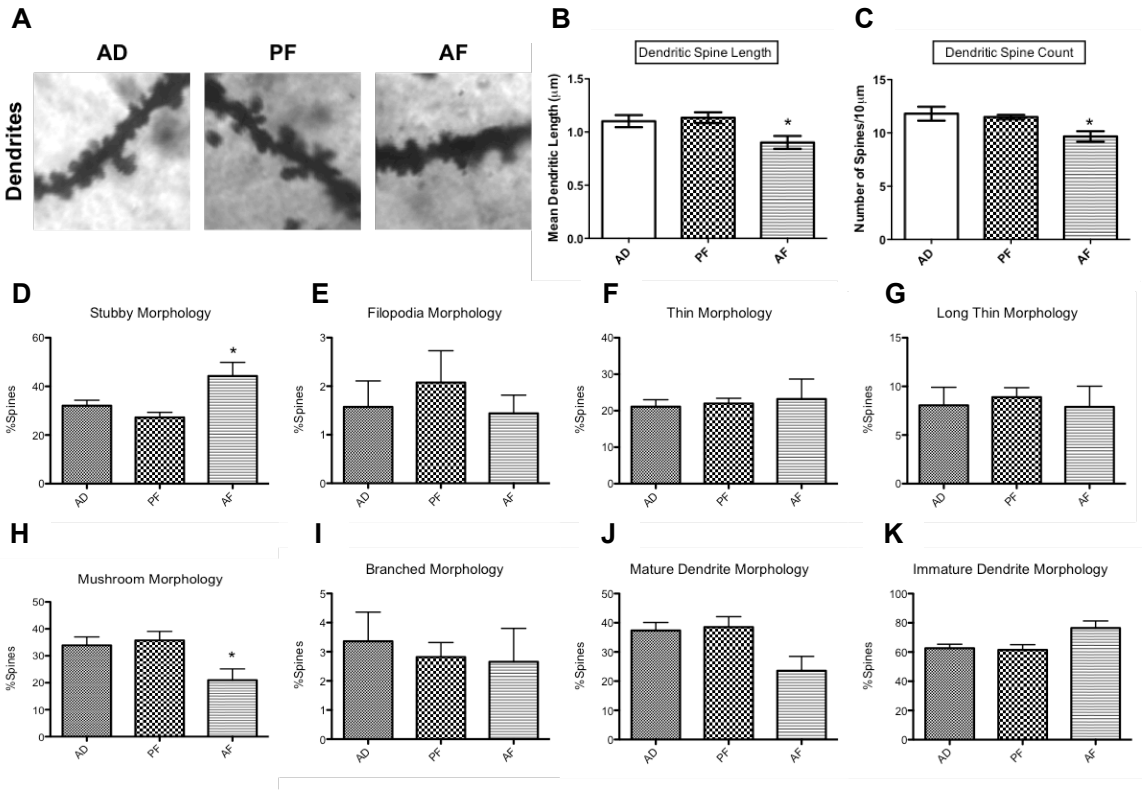


FIGURE 4. POSTNATAL ETHANOL EXPOSURE ALTERS THE NUMBER, LENGTH, AND MORPHOLOGY OF DENDRITES IN THE MBH

The number and length of dendrites in AF-treated pups were reduced. Furthermore, postnatal ethanol exposure increased stubby dendrites and reduced mushroom dendrites. Representative confocal (magnification 100X) images of dendritic spines (a) in the hypothalamus and histograms representing the mean ± SEM of (b) dendritic length and (c) spine number in AD, PF, and AF rat pups on PND 6. Spine morphologies are represented as %spine in histograms for (d) stubby, (e) filopodia, (f) thin, (g) long thin, (h) mushroom, and (i) branched spines. Spine morphologies were then combined into (j) mature and (k) immature morphologies. Data are represented as mean ± SEM (n=4–7).

Currently, the way in which microglia and POMC/β-endorphin neurons interact with each other basally and following fetal ethanol exposure is unknown. Therefore I investigated POMC neuron and microglial interactions via double staining in GFP-POMC neonatal pups following ethanol exposure. Rat neonatal pups were not used due to unsatisfactory results with double staining. These GFP-POMC mice are a transgenic strain that express EGFP so immunohistochemical staining was employed for GFP to visualize POMC neurons and IBA-1 for microglia. Stacked confocal images were used to generate 3D images for analysis of POMC and microglial interactions. Visualization of interactions between POMC and microglia (Fig. 5a) demonstrated that AF-treated rats exhibited a greater ratio of microglial soma interactions with POMC neurons than PF and AD controls; AD and PF POMC neurons had greater interactions with microglial processes than AF (Fig. 5c). Furthermore, AF-treated rats had significantly less POMC neurons in the arcuate nucleus (Fig. 5b, d) as well as relative to PF and AD controls. Taken together, these data demonstrate that postnatal ethanol exposure altered the way microglia interacted with POMC neurons, as well as reduced their quantity in the arcuate nucleus.

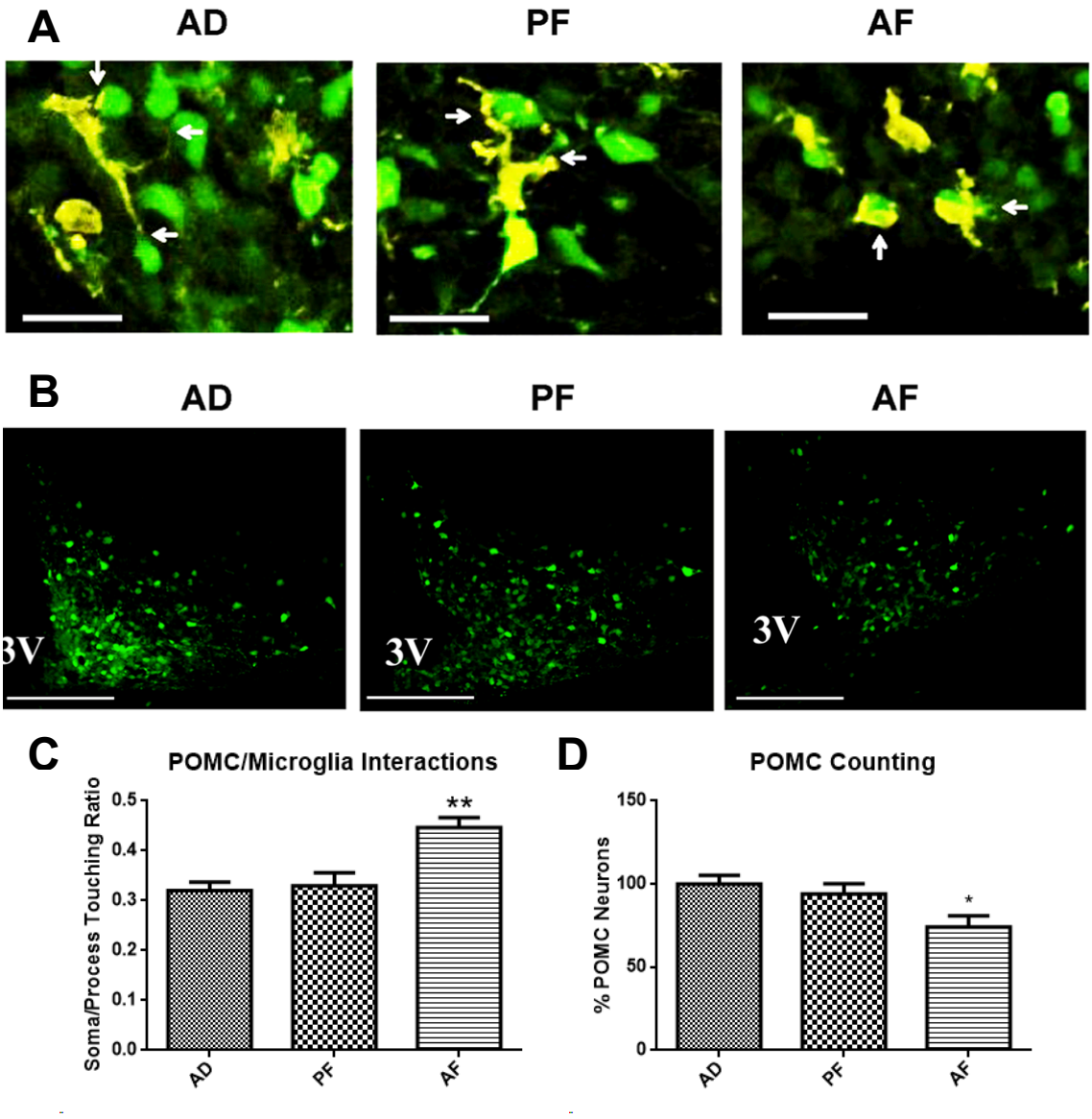


FIGURE 5. POSTNATAL ETHANOL EXPOSURE ALTERS MICROGLIAL/POMC INTERACTIONS AND REDUCES POMC NEURONS

Postnatal ethanol exposure promoted microglial soma interactions with and reduced the number of POMC neurons. Representative images and quantification of IBA-1 positive microglia and GFP-POMC neuronal interactions (a) and a histogram (c) representing the mean ± SEM of soma/process interactions. Representative images of POMC neurons (b) and a histogram (d) representing the mean ± SEM of POMC-positive cells in the arcuate nucleus. Data are represented as mean ± SEM (n=5–7). The differences between AD, PF, and AF were compared by one-way analysis of variance (ANOVA) and the Newman-Keuls posttest. *, $p<0.05$, AF vs PF and AD, **, $p<0.01$ AF vs PF and AD.

Lastly, postnatal ethanol exposure has been shown to activate microglia to release proinflammatory cytokines, alter their morphology, and interact with POMC neurons via their soma. Furthermore, the number of POMC neurons was reduced in the arcuate nucleus of AF-treated rats. To determine whether microglial activation was responsible for the loss of β-endorphin neurons, neonatal pups were treated with minocycline 1 hour before exposure to ethanol for 5 consecutive days. Next, the arcuate nucleus was stained for β-endorphin neurons (Fig. 6) and these neurons were counted. β-endorphin staining in the arcuate nucleus revealed that minocycline administration prevented ethanol's ability to reduce the number of β-endorphin neurons in the arcuate nucleus (Fig. 6). These data illustrate that inhibition of microglial activation completely prevents ethanol-induced loss of β-endorphin neurons.

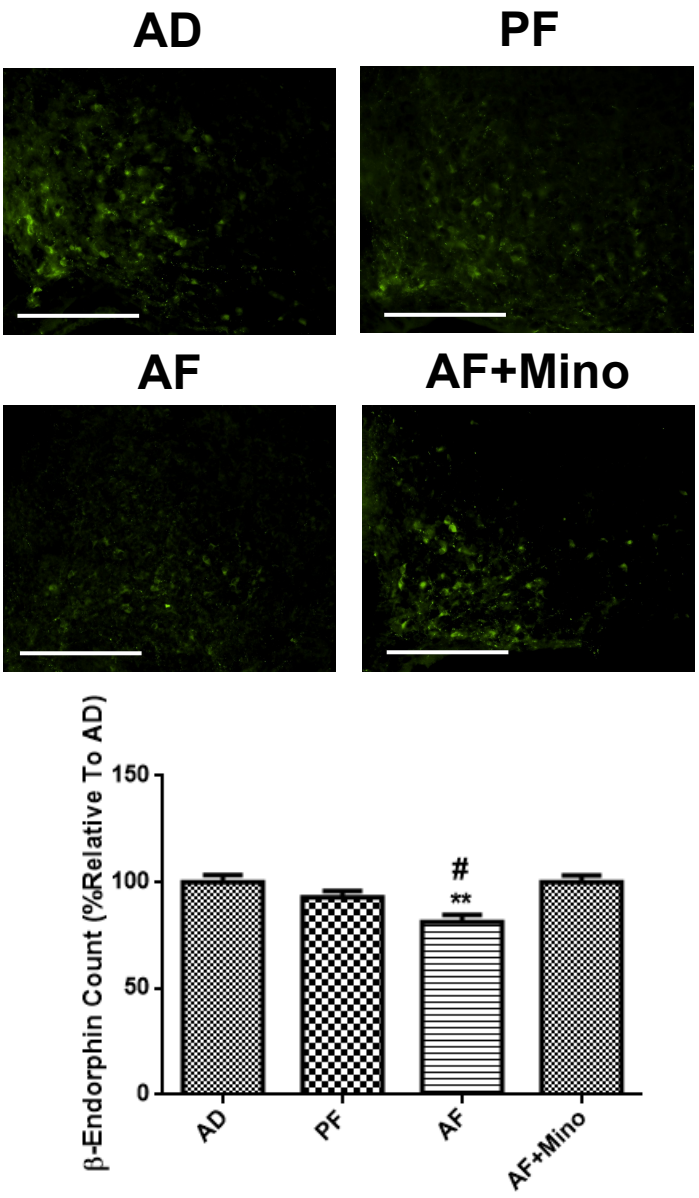


FIGURE 6. MINOCYCLINE ADMINISTRATION PREVENTED LOSS OF β-ENDORPHIN NEURONS IN FETAL ETHANOL EXPOSED NEONATAL RAT PUPS.

Postnatal ethanol exposure activates microglia and reduces β-endorphin neuronal count in the arcuate nucleus. Minocycline, an inhibitor of microglial activation, completely prevents the loss of β-endorphin neurons following fetal ethanol exposure. Above are representative images and histograms of β-endorphin stained cells in the arcuate nucleus. Scale bars in these figures are 200 μm/each Data are represented as mean ± SEM (n=5). The differences between AD, PF, and AF were compared by one-way analysis of variance (ANOVA) and the Newman-Keuls posttest. *, $p<0.05$, AF vs PF, AD, and AF+Mino.

As mentioned previously, β-endorphin is an opioid peptide that can bind to MOR and DOR on microglia. Whether opioid receptors participate in communication between β-endorphin and microglia during ethanol neurotoxicity has yet to be determined. Therefore, I first set out to demonstrate co-localization of DOR and MOR with microglia utilizing immunohistochemistry. Visualization of DOR-positive (Fig. 7a-d) and MOR-positive (Fig. 7e-h) microglia are presented below.

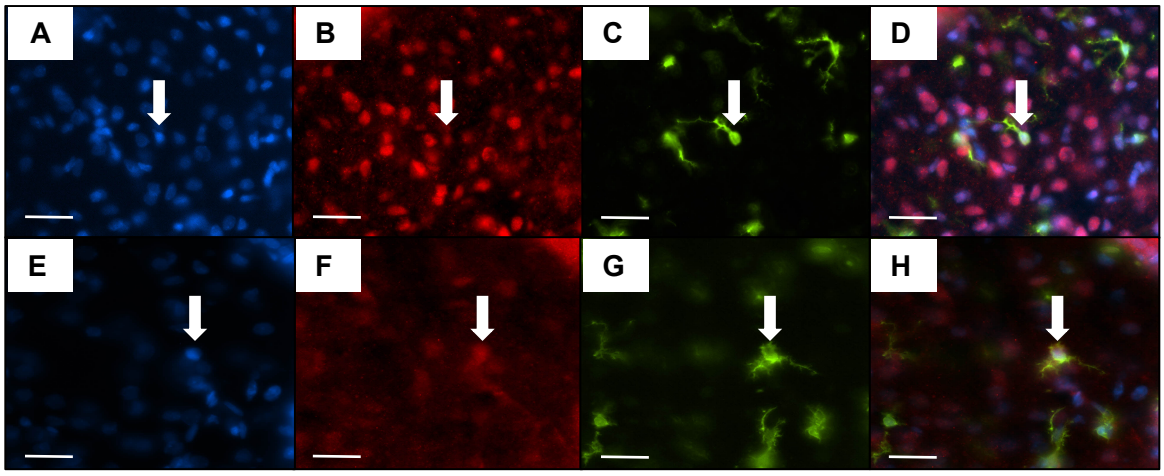


FIGURE 7. MICROGLIA EXPRESS MOR AND DOR

Immunohistochemical evidence the microglia express MOR and DOR. Characterization of IBA-1 and MOR/DOR positive microglia in the arcuate nucleus of postnatal pups. Representative images of (a, e) DAPI, (b) DOR, (f) MOR, and (c, g) IBA-1 stained cells. Representative merged images of (d) DOR and IBA-1 positive microglia and (h) MOR and IBA-1 microglia.

Activation of MOR and DOR by agonists or inhibition via antagonists may differentially regulate microglial function. To quantify whether opioid receptor antagonism affected microglial activation, microglia were isolated using an Optiprep gradient that has produced >90% purity in previous lab experiments from the MBH of postnatal rats (AD/PF/AF) following co-administration of MOR antagonist naltrexone (NTX) or DOR antagonist naltrindole (NTD). Those microglia where then assayed for changes in the expression of receptors that represent microglial activation, as well as MOR and DOR. Preliminary data found no differences between male and female pups therefore we combined sexes for all experiments (Appendix Fig. 1).

First, the results from flow cytometry confirmed the expression of MOR and DOR on microglia (Fig 8c-f). Furthermore, ethanol exposure increased the expression of MOR but not DOR on microglia and these receptor levels were reduced by administration of NTX or NTD, respectively (Fig. 8c-f). Next, AF-treated rats exhibited increased expression of IBA-1 and TLR-4 compared to AD and PF controls, consistent with previous findings of ethanol-induced microglia activation. However, inhibition of MOR with NTX prevented ethanol-induced increases in IBA-1 and TLR-4, returning their concentration to control levels. DOR antagonism did not decrease ethanol-induced activation of these receptors (Fig. 8a, b, g, & h). These data illustrate that microglia express MOR and DOR and theses receptors are downregulated by NTX and NTD, respectively. Furthermore, postnatal ethanol exposure activated microglia, as demonstrated by augmentation of IBA-1 and TLR-4 fluorescent intensity on microglia. Importantly, microglia activation was inhibited by MOR antagonism, demonstrating that MOR is involved in ethanol-induced microglial activation and DOR antagonism is not.

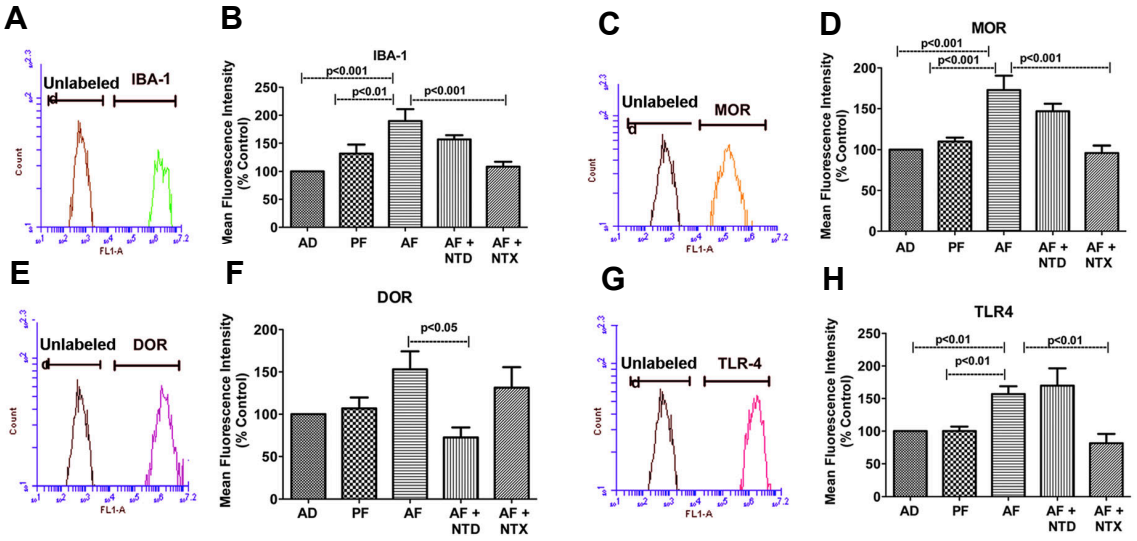


FIGURE 8. POSTNATAL ETHANOL EXPOSURE DIFFERENTIALLY EFFECTS MICROGLIAL OPIOID RECEPTORS EXPRESSION

Postnatal ethanol exposure augments IBA-1, MOR, and TLR-4 proteins in microglia. MOR but not DOR antagonism reverses this effect. Histograms show representative fluorescent intensities of isolated microglia for each protein compared to unstained or unlabeled control cells. Bar graphs show mean fluorescence intensity of staining of IBA-1 (A, B), MOR (C, D), DOR (E, F), and TLR-4 (G, H). p-values are presented in the figures to show post-hoc differences between treatment groups.

The data presented above (Fig. 8) demonstrated an increase of TLR-4 on microglia following postnatal ethanol exposure. To confirm a functional outcome for the upregulation of TLR-4, downstream proinflammatory signaling molecules were assayed from isolated microglia. AF-treated rats exhibited upregulation of p-38 MAPK, p-JNK, p-Akt, and NF-κB (Fig. 9a-h) in isolated microglia. Increased activation of these molecules has been shown to promote production of proinflammatory cytokines in microglia. Therefore, postnatal ethanol exposure activated TLR-4 dependent, proinflammatory cytokine signaling, but MOR antagonism blocked it. Thus, MOR activation is necessary for ethanol-induced TLR-4 dependent signaling. NTD did not affect TLR-4 signaling molecules so DOR is not involved in ethanol-induced TLR-4 dependent signaling.

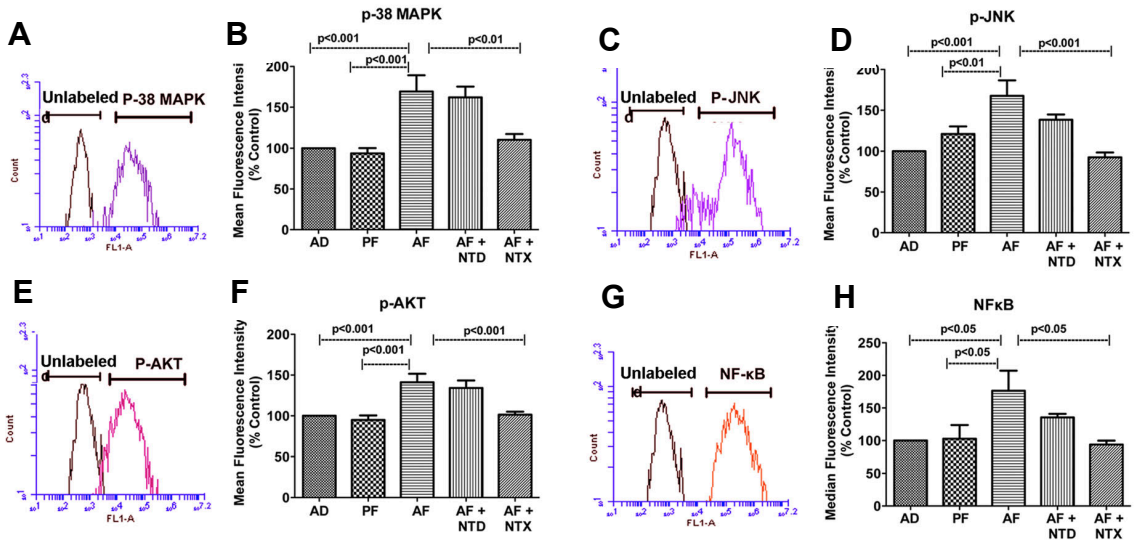


FIGURE 9. MOR ANTAGONISM PREVENTS ETHANOL-INDUCED TLR-4 SIGNALING

Postnatal ethanol exposure augments p-38 MAPK, p-JNK, p-Akt, and NF-κB proteins in microglia. MOR but not DOR antagonism reversed this effect. Histograms show representative staining of isolated microglia for each protein compared to unstained or unlabeled control cells. Bar graphs show mean fluorescence intensity of staining of p-38 MAPK (a, b), p-JNK (c, d), p-Akt (e, f), and NF-κB (g, h). p-values are presented in the figures to show post-hoc differences between treatment groups.

Lastly, since MOR was upregulated on microglia and inhibition of MOR, but not DOR, prevented a TLR-4-dependent proinflammatory-signaling cascade, I investigated whether MOR and DOR contributed to ethanol-induced neurotoxicity and apoptosis of β-endorphin neurons. Cell of the arcuate nucleus were stained for β-endorphin in AD-, PF-, AF-, AF+NTX-, and AF+NTD-treated neonatal rat pups and representative images can be found below (Fig. 10). Counting of β-endorphin positive neurons revealed that while AF-treated animals had a reduction of β-endorphin neurons, co-administration of NTX prevented this loss. Furthermore, antagonism of DOR with NTD did not prevent the loss of β-endorphin neurons when administered in AF rats.

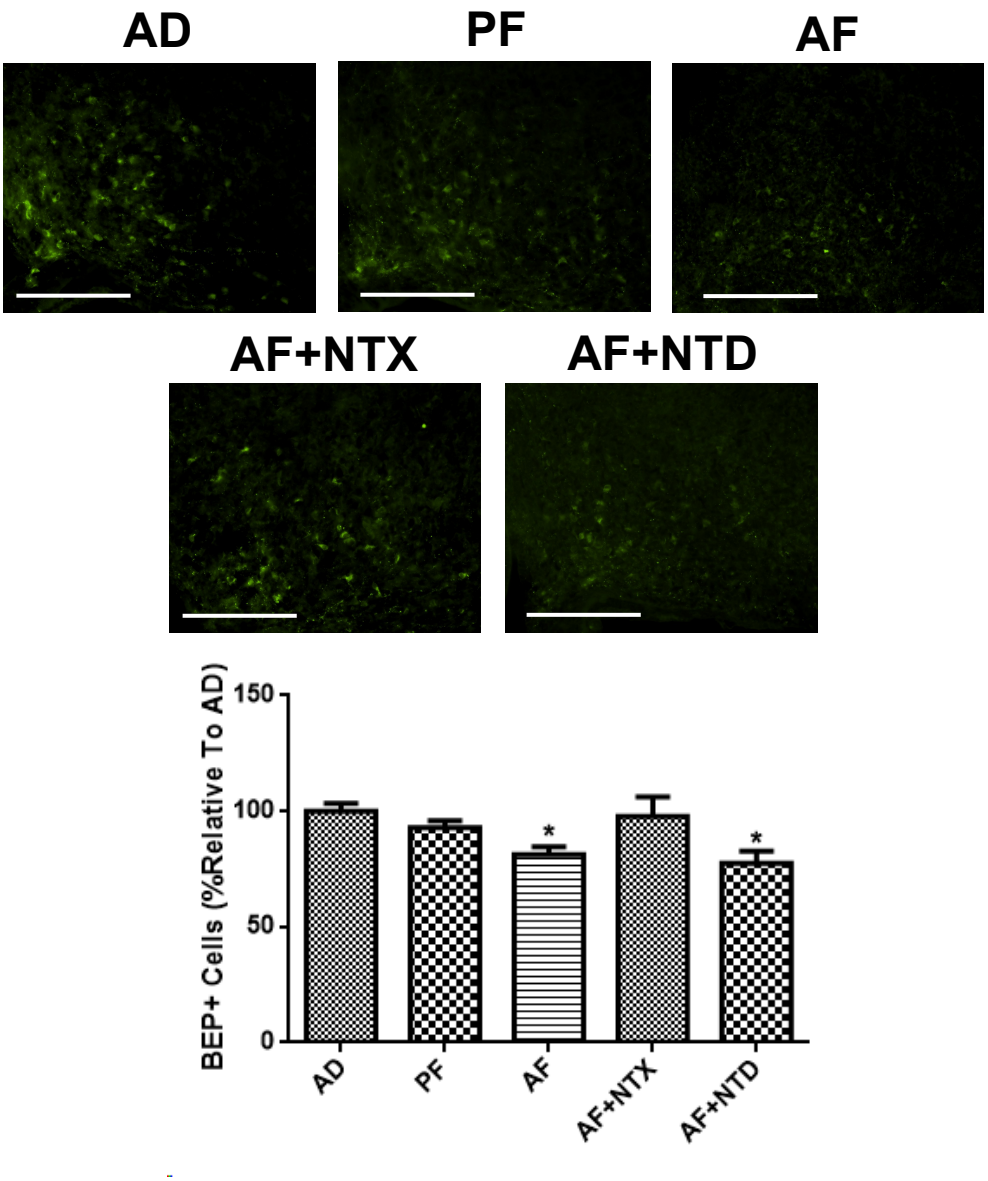


FIGURE 10. MOR BUT NOT DOR ANTAGONISM PREVENTED MICROGLIAL-INDUCED β-ENDORPHIN NEURONAL LOSS

Postnatal ethanol exposure reduces the number of β-endorphin neurons in the arcuate nucleus. Naltrexone, an inhibitor of MOR, completely prevented the loss of β-endorphin neurons in AF-treated animals but naltrindole, a DOR antagonist, did not. Above are representative images and histograms of β-endorphin stained cells in the arcuate nucleus. Scale bars in these figures are 200 μm/each Data are represented as mean ± SEM (n=5). The differences between AD, PF, AF, AF+NTX, and AF+NTD were compared by one-way analysis of variance (ANOVA) and the Newman-Keuls posttest. *, $p<0.05$, AF and AF+NTD vs AD, PF, and AF+NTX.

2.4 DISCUSSION

Postnatal ethanol exposure induces apoptosis of β-endorphin neurons *in vitro* and *in vivo*, resulting in dysregulation of the HPA axis (Chen et al., 2006a; Logan et al., 2015). However, the mechanism involving the loss of β-endorphin neurons is not completely understood. In this study, I demonstrated that a third trimester equivalent exposure to ethanol increased the expression of proinflammatory cytokines in the MBH, where β-endorphin neurons are located. Furthermore, postnatal ethanol exposure increased the number of microglia in the arcuate nucleus, as well as the amount of partially and fully activated microglia. Activation of microglia is generally accompanied by increased release of proinflammatory cytokines, such as TNF-α, to induce apoptosis of neurons. Increased activation of microglia, as observed in Alzheimer's disease, can lead to augmented synaptic pruning. Along with that I found that postnatal ethanol exposure decreased the number of dendritic spines, as well as altered their morphology towards more immature, shorter dendrites. These changes could be associated with dyregulation of neuronal signaling in the hypothalamus and possible deficits such as anxiety disorders and stress abnormalities (Mathew et al., 2008). Furthermore, activated microglia are also associated with increased phagocytosis (Saito et al., 2016). In concert, I found that postnatal ethanol exposure increased the ratio of microglial soma interactions with POMC compared to AD and PF controls. Furthermore, β-endorphin neuronal cell count was reduced in AF-treated rats relative to their controls. Taken together, this suggests that the increased soma interactions by microglia may in fact be increased phagocytosis of β-endorphin neurons, which, in addition to proinflammatory cytokine release, could be the cause of reduced β-endorphin cell counts in AF-treated rats. In order to confirm that

microglial activation was inducing the loss of β-endorphin neurons, I administered minocycline to AF-treated rats. While it has been shown that minocycline is not a specific inhibitor of microglia, it can still significantly reduce microglial activation (Möller, et al., 2016). Administration of minocycline completely prevented the loss of β-endorphin neurons in the arcuate nucleus of AF+Mino treated rats, suggesting that activated microglia were promoting the loss of β-endorphin neurons.

β-endorphin is an opioid peptide and may be used to communicate with microglia via MOR and DOR to regulate homeostasis. To determine whether opioid receptors regulate microglial activation I first confirmed that microglia, isolated from the MBH, contained MOR and DOR. Indeed, MBH-isolated microglia express MOR and DOR, which is in concordance with previous studies (Debrenis et al., 1999; Turchan-Cholewo et al., 2008; Horvath et al., 2009). Next, microglia that were postnatally exposed to ethanol exhibited upregulation of IBA-1 and TLR-4 protein, which are classic markers for microglial activation. Interestingly, MOR antagonist NTX completely blocked ethanol-induced augmentation of IBA-1 and TLR-4, suggesting that MOR antagonism can diminish microglial activation. DOR antagonism with NTD did not prevent microglia activation. Furthermore, ethanol exposed rat neonates also exhibited increased MOR but not DOR expression in microglia and these receptor levels were reduced following co-administration of NTX or NTD, respectively. Since postnatal ethanol exposure upregulated TLR-4 protein levels in microglia, downstream TLR-4 signaling molecules were assayed to determine whether this change induced a functional outcome. Postnatal ethanol exposure increased p-Akt, NF-κB, p38 MAPK, and p-JNK. Phosphorylated-Akt is the active form of Akt and can phosphorylate nuclear factor of kappa light polypeptide

gene enhancer in B-cells inhibitor, alpha (IκBα), which causes the release of NF-κB and allows it to translocate into the nucleus to stimulate the production of proinflammatory cytokines (Bai et al., 2009). Phosphorylated-JNK and p38 MAPK can activate the AP-1 transcription factor, which can also upregulated transcription of proinflammatory cytokines (Lu et al., 2008). This effect was attenuated by NTX and not NTD administration. This suggests that MOR also play are also involved in downstream TLR-4 signaling to induce the transcription of proinflammatory cytokines, another hallmark of microglial activation. Lastly, to resolve whether opioid receptors on microglia affect ethanol-induced loss of β-endorphin neurons I stained the arcuate nucleus of postnatal exposed rats. I found that AF+NTX completely prevented ethanol-induced reductions of β-endorphin neurons, while AF+NTD still produced decreased β-endorphin neuron counts.

In summary, the data presented above strongly suggests that ethanol-induced apoptosis of β-endorphin neurons is the result of microglial activation, as well as the differential action of microglial opioid receptors. Postnatal ethanol exposure activated microglial, induced changes in morphology towards partially and fully amoeboid, and augmented the production of proinflammatory signals to kill β-endorphin neurons. TLR-4 activation in AF-treated microglia also promoted a proinflammatory phenotype but was inhibited by NTX. In addition, NTX also prevented β-endorphin apoptosis, suggesting that MOR signaling is critical regulator of microglia activation and β-endorphin apoptosis. This is consistent with *in vitro* data presented in Shrivastava et al. where MOR agonist DAMGO increased microglial p38 MAPK and p-Akt, as well as proinflammatory cytokine release including TNF-α (Shrivastava et al., 2017). On the other hand DOR

antagonism did not prevent microglial activation or β-endorphin apoptosis. It was suggested in Shrivastava et al. that DOR plays a neuroprotective role inhibiting ethanol-induced increases in microglial p38 MAPK, p-Akt, and cytokine production including TNF-α *in vitro* following co-administration of DPDPE, a DOR agonist, and ethanol (Shrivastava et al., 2017). My data does not disagree with this assertion and may even support it since DOR antagonism prevented a neuroprotective effect in AF microglia.

CHAPTER 3

3.1 INTRODUCTION

Exosomes are small vesicles (30-150 nm in diameter) that form from the inward budding of the limiting membrane of endosomes. Due to inward budding, exosomes contain proteins, RNA, lipids, and extracellular markers from their cells (Colombo et al., 2014). Once formed, exosomes can travel to nearby or distant cells and be taken up through a number of mechanisms including membrane fusion, receptor-mediated endocytosis, and phagocytosis (Mulcahy et al., 2014). Once exosomes enter the target cell their cargo is released into the cytoplasm and can alter cellular homeostasis. This form of signaling allows cells to communicate with each other via exosomes Moreover, Fauré et al. were the first to demonstrate that neurons could release exosomes (Fauré et al., 2006). Furthermore, Chivet et al. showed that exosomes released from cultured hippocampal neurons could be taken up by neurons (Chivet et al., 2014).

In addition to serving as a form of communication between neurons, exosomes can be exchanged between neurons and microglia. Bahrini et al. discovered that exosomes, collected from depolarized neurons, could induce microglia to prune more synapses of co-cultured neurons compared to non-exposed microglia (Bahrini et al., 2015). Other experiments have found that serotonin and ATP released from neurons can bind to microglial receptors and induce the release of extracellular vesicles with metabolic and immune functions (Glebov et al., 2015; Drago et al., 2017). While neurons can use exosomes to communicate with microglia to maintain homeostasis, neuroinflammation and other neurodegenerative diseases can hijack this system to spread apoptotic factors and induce cell death.

In Alzheimer's disease microglia play an important role in clearing amyloid β-plaque (Aβ) buildups through phagocytosis. However, in a murine model of Alzheimer's disease, microglia can become reactive and produce extracellular vesicles containing those neurotoxic Aβ plaques, send them to neighboring cells, and induce apoptosis (Wang et al., 2012; Joshi et al., 2014). Importantly, inhibition of exosome production with GW4869, a sphingomyelinase inhibitor, reduced Aβ load and microglial activation, which ameliorated apoptosis (Wang et al., 2012). In Parkinson's disease, alpha-synuclein is a major component of Lewy bodies, which can form protein clumps and produce neurotoxicity. Alpha-synuclein has also been shown to induce the release of microglial exosomes containing alpha-synuclein (Chang et al., 2013). These microglial exosomes can travel in the cerebrospinal fluid to nearby or distant cells and induce neurotoxicity (Stuendl et al., 2016). LPS-induced inflammation can also activate microglia to release extracellular vesicles containing IL-1β, pro-IL-1β, and caspase-1 (Bianco et al., 2005). Interestingly, caspase-1 can cleave pro-1L-1β into the active IL-1β peptide. Therefore, activated microglia can release exosomes with apoptotic peptides as well as generate them via enzymatic cleavage of pro-peptides.

Ethanol exposure has also recently been shown to induce the release of exosomes. Exosomes from hepatocytes exposed to ethanol had augmented levels of miRNA-122. This miRNA increased the secretion of proinflammatory cytokines from recipient monocytes, as well as sensitized them to LPS (Momen-Heravi et al., 2015). Furthermore, exosomes from these treated monocytes activated naïve monocytes via miR27a, leading to increased proinflammatory cytokine production and phagocytic activity (Saha et al., 2016). Cho et al. found that binge ethanol exposure increased the number of plasma

extracellular vesicles, as well as the concentration of CYP2P1 and other related p450 proteins in rat exosomes. Exposure of these extracellular vesicles to primary hepatocytes or human hepatoma cells promoted apoptosis through p-Jun, Bax, and cleaved caspase-3 mechanisms (Cho et al., 2017). Taken together the data demonstrate a role for ethanol exposure increases the number of extracellular vesicles from liver and immune cells, which contain apoptotic proteins and miRNAs. Exposure of cells to these exosomes can induce apoptosis.

Since ethanol exposure can activate immune cells, as well as other cells, to produce extracellular vesicles that contain pro-apoptotic cargo, I determined whether postnatal ethanol exposure induces apoptosis of β-endorphin neurons through the release of pro-apoptotic exosomes. Postnatal ethanol exposure also activates microglia, the immune cells of the brain, to induce apoptosis of β-endorphin neurons. Therefore, I also evaluated the role microglial activation played in the activity and cargo of microglial exosomes. Lastly, since opioid receptors are involved in ethanol-induced activation of microglia and apoptosis of β-endorphin neurons, I determined whether they contributed to exosome cargo and activity.

.

3.2 MATERIALS AND METHODS

Animal Use

Animal care and treatment were performed in accordance with institutional guidelines and complied with the National Institutes of Health policy and Rutgers Animal Care and Facilities Committee. Adult Sprague-Dawley rats, obtained from Charles River Laboratories (Wilmington, MA) were kept under 12 hour light/12 hour dark conditions, provided water and rodent chow ad libitum, and mated. Neonates were used for postnatal ethanol exposure experiments.

Postnatal Ethanol Exposure Model

Postnatal pups (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 isocaloric control (PF), or they were left in the litter with the mother (AD). Gavage feeding was conducted at 10:00 AM and 12:00 PM from PND 2-6. After feeding, these pups were immediately returned to the litter. Additionally, some animals were treated subcutaneously with minocycline (45μg/kg; one hour prior to the first feeding), or mu-opioid receptor antagonist naltrexone (NTX, 10mg/kg; 15 minutes prior to the first feeding), or delta-opioid receptor antagonist naltrindole (NTD, 10mg/kg; 15 minutes prior to the first feeding) or neutral sphingomyelinase inhibitor GW4869 (GW4869, 2.5mg/kg; 15 minutes prior to the first feeding). All of these drugs were purchased from Sigma Aldrich (St. Louis, MO). Two hours after the last feeding on PND 6, some of the pups were transcardially perfused with 4% paraformaldehyde, postfixed overnight, cryoprotected in 30% sucrose, and cut into 30μm coronal sections

for immunohistochemistry. Additional pups were sacrificed and their MBH were collected for exosome isolation, Western blot, and aminopeptidase (CD13) activity assay.

Exosome Isolation

Rat MBH, as previously described with modifications (Shrivastava et al., 2017), were dissected and placed in cold Hank's balanced salt solution (HBSS) media (Sigma Aldrich, St. Louis, MO). Then MBH were mechanically dissociated in 5mL syringes using 18-gauge needles followed by 21-gauge needles. The homogenate was filtered through 40μm cell strainers and then centrifuged at 3000 x g for 10 minutes at 4°C to remove cellular debris. Each supernatant was then passed through a 0.2μm filter. Next, the samples were centrifuged serially at 4°C: 300 x g for 10 min, 2000 x g for 10 min, and 10,000 x g for 30 min. An equal amount of Exosome Isolation Reagent (Thermo Fisher Scientific; Waltham, MA) was added to each supernatant and incubated overnight at 4°C. On the next day, the mixtures were centrifuged at 10,000 x g for 60 min at 4°C and the exosome pellets were collected. The pellets were suspended in 50 μl PBS and two equal aliquots were prepared, one for Western blot and one for CD13 aminopeptidase activity assay, transmission electron microscopy, and nanoparticle tracking analysis. For Western blot, resuspended exosomes were prepared in Exosome Resuspension Buffer (Total Exosome RNA and Protein Isolation Kit, Thermo Fisher Scientific; Waltham, MA). Nanoparticle tracking analysis (NTA) was performed on intact exosomes using a NanoSight Range (NanoSight Ltd., Malvern, Westborough, MA, USA) equipped with a 405 nm laser and an automatic syringe pump system. Samples were diluted 1:1000 in PBS and

CD13 Aminopeptidase Activity Assay

Exosomes were mixed with 200 mmol of leucine-p-nitroaniline (Sigma Aldrich, St. Louis, MO) in a 96-well plate (20 ug/ml total exosomal protein in 150 ml of PBS/well). The release of p-nitroaniline at 60, 120, 240, and 360 min was used to follow aminopeptidase activity by measuring the absorbance at 405 nm.

Immunohistochemistry

Serial coronal sections of 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, one PF, and one AF brain section. The sections were washed in PBS twice followed by antigen retrieval in citrate buffer (pH 6.2) at 100°C for 10 min. After two washes in PBS-T (0.05% Triton-X) the sections were incubated in blocking buffer (2.5% normal horse serum in PBS-T) at room temperature for 30 min. The sections were subsequently incubated overnight at 4 °C with the rabbit anti-ß-endorphin (1:1000, Peninsula Laboratories, San Carlos, CA). After the primary antibody incubation samples were washed in PBST and then sections were incubated with an Alexa Fluor 488 donkey anti-rabbit secondary antibody (1:500, Thermo Fisher Scientific, Grand Island, NY). 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).

Western Blot

Exosome samples were processed for protein extraction followed by the Bradford Assay to quantify total protein levels (Bio-Rad Laboratories, Hercules, CA). 15 µg of exosome total protein or liver protein, were run in 4-20% SDS PAGE and transferred to Polyvinylidene fluoride (PVDF) membranes at 30 V overnight at 4°C. The membranes were blocked in 5% non-fat milk in tris-buffered saline (TBS) at 4°C for 5 hours. The membranes were incubated with primary antibody in the same blocking buffer with 0.2% Tween-20 at 4°C overnight. The following primary antibodies were used for Western blot: rabbit anti-CD13, rabbit anti-cleaved caspase-3, rabbit anti-calnexin, rabbit anti-cytochrome c (1:200; Cell Signaling Technology, Danvers, MA), mouse anti-C1q (1:200; Abcam, Cambridge, MA), and mouse anti-C3, mouse anti-CD63, and rabbit anti-GM130 (1:100; Santa Cruz Biotechnology; Dallas, TX). Next, the membranes were washed in TBST (PBS or TBS with 0.1% Tween-20) and then incubated with HRP secondary antibodies at room temperature for 90 minutes. The membranes were washed and developed on film. The protein band intensities were determined by Image Studio Lite (LI-COR Biotechnology, Lincoln, NE) and normalized with corresponding CD63 band intensities.

Statistical Analysis

Statistical analyses were conducted using Graph Pad Prism 6.0 software for Mac (Graph Pad Software, Inc., San Diego, CA). I compared treatment groups by one-way and two-way ANOVAs with Newman-Kuels post-hoc tests. A value of $P<0.05$ was considered significant.

3.3 RESULTS

As mentioned previously, exosomes are small vesicles (30-150 nm in diameter) that form from the inward budding of the limiting membrane. Therefore, I first assayed the exosomes I collected to confirm that their size fell within the range of 30-150 nm. Nanoparticle tracking analysis determined that *in vivo* MBH exosomes had a mean particle diameter of 138.7 nm, a mode of 82 nm, and a concentration of $2.78*10^{11}$ particles/mL (Fig. 11a). A visualization of exosomes during nanoparticle tracking analysis can also be seen below (Fig. 11b). It should be noted that exosomes appeared to be clumped together, forming distortions in the video and may be reason why larger particle sizes were observed during nanoparticle tracking analysis. Electron microscopy was performed to confirm the size and morphology of MBH exosomes. MBH exosomes were within the 30-150 nm ranges and had the characteristic cup-shape typically observed in exosomes under transmission electron microscopy (Fig. 11c). Lastly, MBH exosomes were assayed by Western blot to confirm their purity against rat liver lysate, which served as a positive control. These exosomes were positive for exosome marker protein CD-63 (Fig. 11d) and were negative for possible vesicular contaminates cytochrome c (mitochondrial-specific protein), GM130 (Golgi apparatus-specific protein), and calnexin (endoplasmic reticulum-specific protein). Therefore, these data demonstrate that the extracellular vesicles collected from MBH tissue are exosomes.

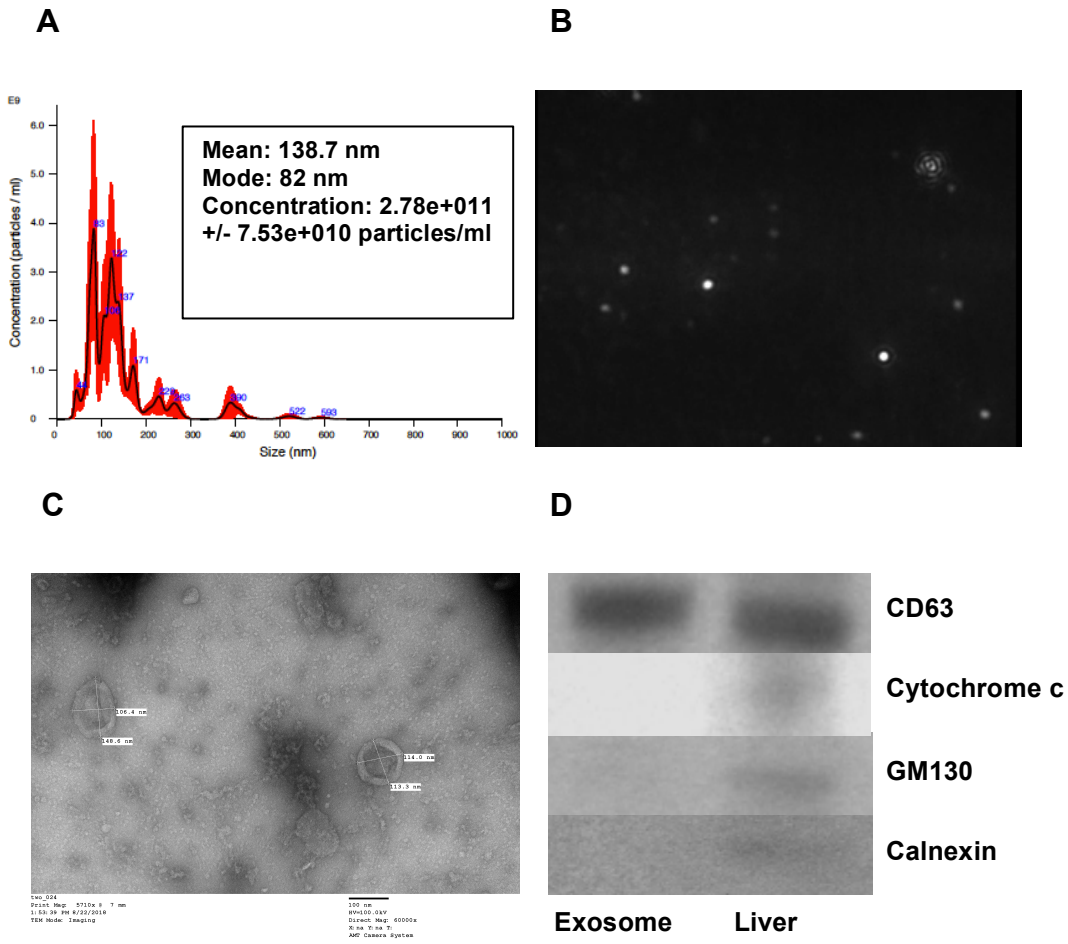


FIGURE 11. CHARACTERIZATION OF MBH EXOSOMES

Nanoparticle tracking analysis, transmission electron microscopy, and Western blot verification of exosomes from postnatal rats PD6. For Nanoparticle Tracking Analysis, the calculated size distribution is depicted as a mean (black line) with standard error (red shaded area). (a) Mean particle size, mode particle size, and concentration of particles collected from MHB exosomes are shown. (b) Visual representation of exosomes recorded by Nanoparticle tracking analysis. (c) Representative image of exosomes under transmission electron microscopy. The width and length of each exosome is represented on the individual exosome. (d) Representative bands for the protein levels of exosome marker CD63, Mitochondria marker Cytochrome c, Golgi Apparatus marker GM130, and Endoplasmic Reticulum marker Calnexin found in MBH exosome and liver lysates.

Next, since exosomes can transport cargo from one cell to another and induce apoptosis, I utilized Western blot to determine whether postnatal ethanol exposure altered the cargo of MBH exosomes. MBH exosomes from AF-treated rats exhibited augmented microglial-specific protein, CD13, relative to AD controls (Fig. 12a). Next I found that pro-apoptotic cleaved caspase-3 (CC3) and C1q were elevated in AF-treated MBH exosomes (Fig. 12b, c). Representative Western blot bands are also represented below. Taken together, these data show that postnatal ethanol exposure increased the amount of microglial exosomes and these exosomes had augmented quantities of pro-apoptotic peptides.

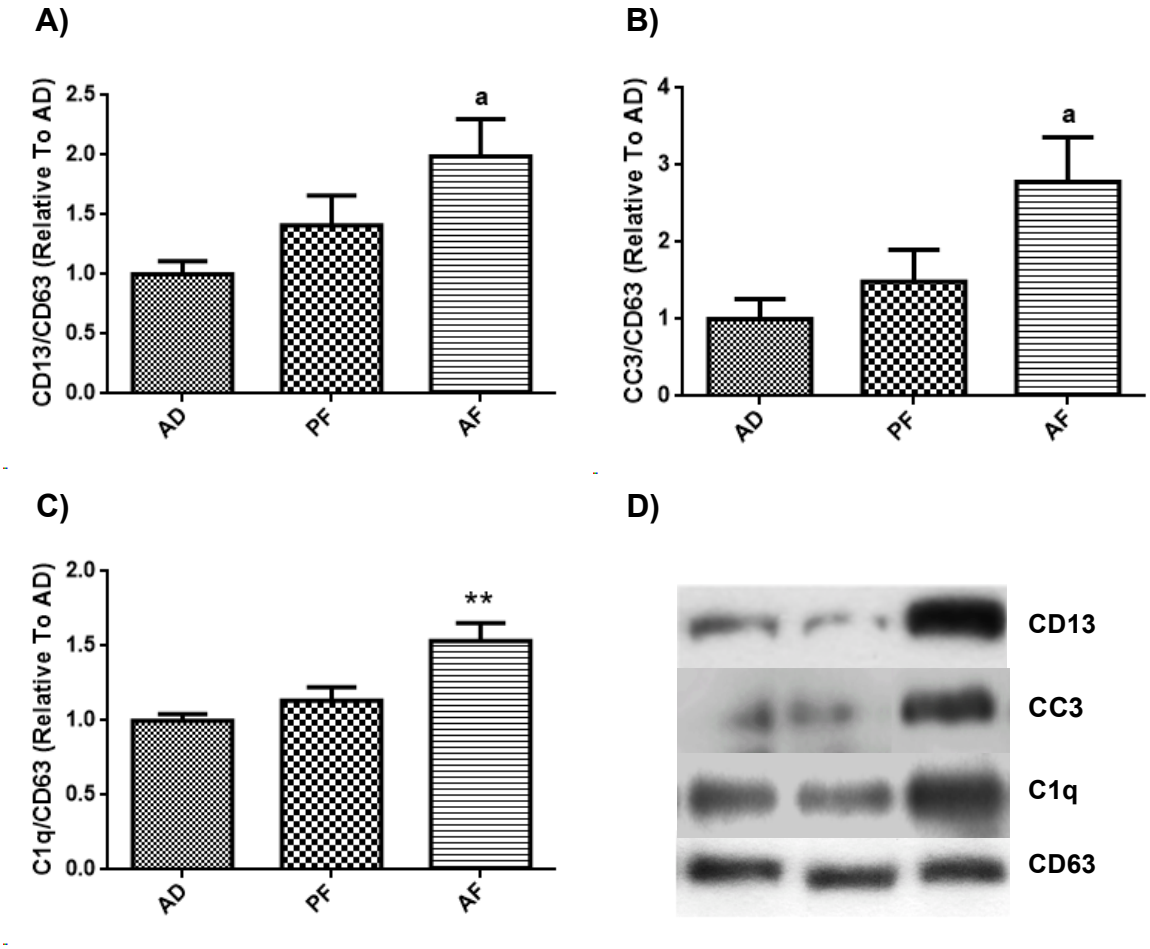


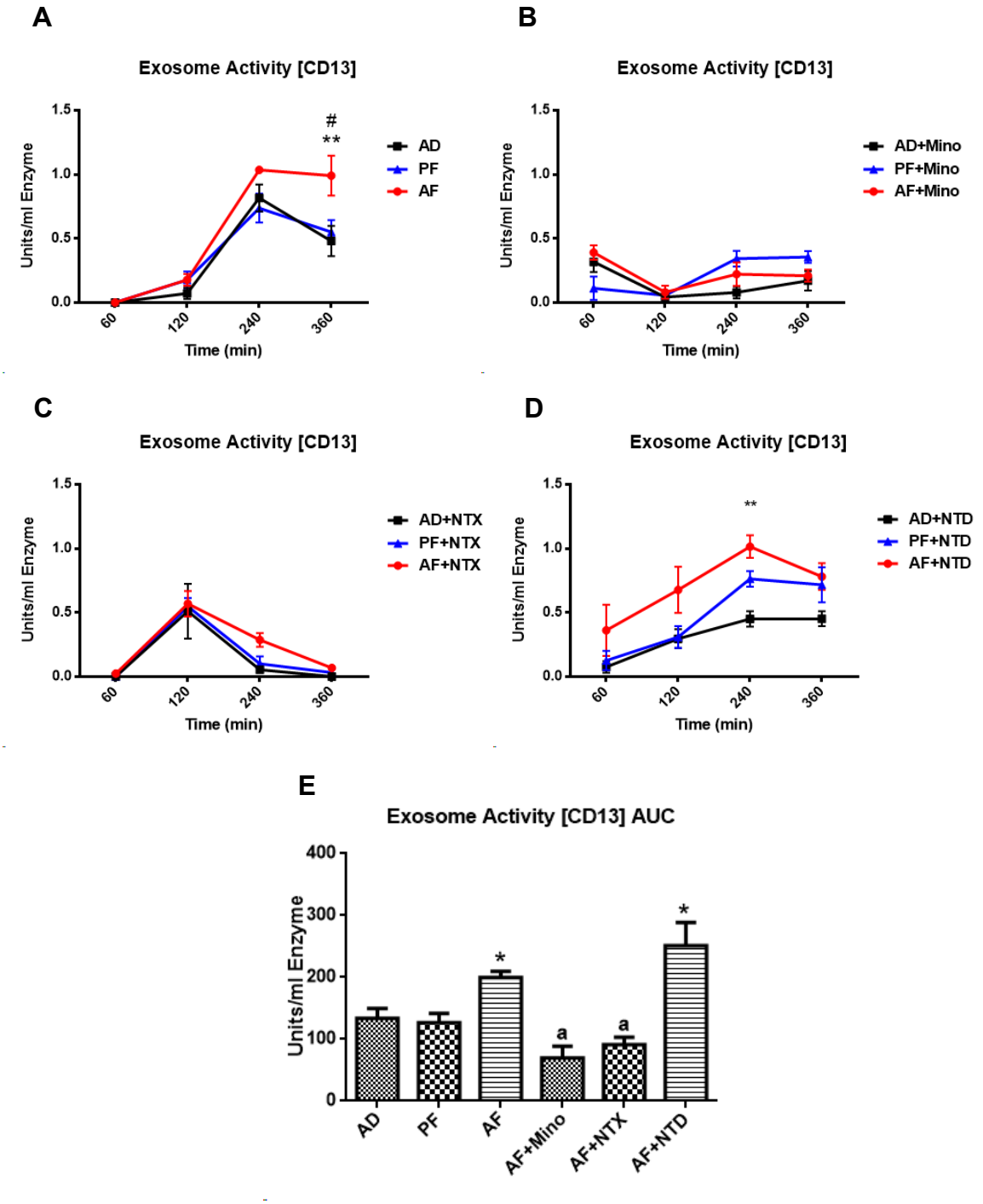
FIGURE 12. POSTNATAL ETHANOL EXPOSURE INCREASES PROAPOTOTIC AND MICROGLIAL SPECIFIC PROTEINS IN MBH EXOSOMES

The effect of postnatal ethanol exposure on exosomes collected from the mediobasal hypothalamus. The protein levels were measured by Western blot. Bar graphs representing the protein levels of pro-apoptotic factor (a) cleaved caspase-3, (b) C1q, and (c) microglial-specific marker CD13 along with representative bands for AD, PF, and AF exosome samples. Data are presented as mean ± SEM (n=5-6). The differences between AD, PF, and AF were compared by one-way analysis of variance (ANOVA) and the Newman-Keuls posttest. **$p<0.01$, AF vs PF and AD controls, a, $p<0.05$ AF vs AD controls.

CD13, also called aminopeptidase, is enzymatically active on microglial-derived exosomes. Therefore, to confirm the increase of CD13 protein found on AF-treated exosomes, I quantified exosomal CD13 activity. In concert with the Western blot data, AF-treated rats exhibited significantly greater CD13 activity than AD and PF controls (Fig. 13a, e). Since activation of microglia has been shown to increase the release of exosomes, I determined whether microglial activation was required for ethanol-induced changes in CD13 activity. Minocycline administration completely attenuated CD13 activity in AF-treated rats (Fig. 13b, e). Thus, postnatal ethanol exposure activates microglia to release exosomes. Because opioid receptors also regulate microglial activation I determined whether MOR and DOR antagonism differentially regulated CD13 activity. In concordance with previous data, NTX nullified ethanol-induced increases in CD13 activity and NTD did not (Fig. 13c-e). Therefore, inhibition of ethanol-induced microglial activation through minocycline or NTX administration attenuates the release of microglial exosomes.

FIGURE 13. INHIBITION OF MICROGLIA ACTIVATION AND MU-OPIOID RECEPTOR ANTAGONISM SUPPRESS CD13 ACTIVITY IN ETHANOL-EXPOSED NEONATES

Presented here are the effects of ethanol exposure, inhibition of microglial activation, and opioid receptor antagonism on CD13 activity. Aminopeptidase activity of exosomes collected from the mediobasal hypothalamus at 4 times points (60, 120, 240, and 360 minutes) for (a) AD, PF, and AF, (b) minocycline, (c) naltrexone, and (d) naltrindole treated pups, along with area under the curve (e) histograms for AD, PF, AF, AF+Mino, AF+NTX, and AF+NTD. Data are presented as mean ± SEM. The differences between treatment groups were compared by two-way analysis of variance (ANOVA) and the Newman-Keuls posttest. *, $p < 0.05$, AF and AF+NTD vs AD and PF, **, $p < 0.01$ AF vs AD, a, $p < 0.05$ AF+Mino and AF+NTX vs AF, #, $p < 0.01$ AF vs PF.


A
Exosome Activity [CD13]
Units/ml Enzyme
Time (min)
AD
PF
AF
#
**
B
Exosome Activity [CD13]
AD+Mino
PF+Mino
AF+Mino
C
Exosome Activity [CD13]
AD+NTX
PF+NTX
AF+NTX
D
Exosome Activity [CD13]
AD+NTD
PF+NTD
AF+NTD
**
E
Exosome Activity [CD13] AUC
AD
PF
AF
AF+Mino
AF+NTX
AF+NTD
a
a
*
*

Next, since microglial exosome activity was altered by inhibition of microglial activation and opioid antagonism, I determined whether these treatments would also alter MBH exosome cargo. First, as previously demonstrated, AF-treatment produced greater CD13 expression than AD controls and cleaved caspase-3 and C1q expression was greater than AD and PF controls (Fig. 14a-c). NTX and minocycline administration attenuated microglial CD13 activity and CD13 protein expression on MBH exosomes from AF-treated rats. In concordance with this decrease in CD13-positive exosomes, NTX and minocycline treatment also prevented ethanol-induced augmentation of pro-apoptotic cleaved caspase-3 and C1q protein expression in AF-treated MBH exosomes. NTD administration did not prevent ethanol-induced changes in these exosomes; in fact, AF+NTD had greater C1q expression than AD, PF, AF+Mino, and AF+NTX. Thus, inhibition of ethanol-induced microglial activation with NTX or minocycline decreased the release of microglial specific exosomes and reduced the cleaved caspase-3 and C1q from those exosomes.

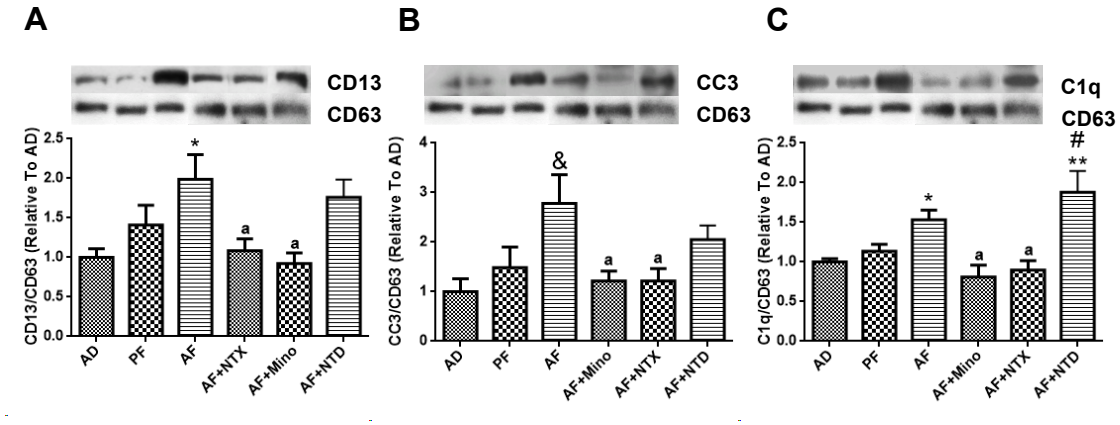


FIGURE 14. POSTNATAL ETHANOL EXPOSURE EFFECT ON MBH EXOSOMES IS REVERSED BY MICROGLIAL INACTIVATION THROUGH MU-OPIOID RECEPTOR ANTAGONISM OR MINOCYCLINE ADMINISTRATION

The effect of inhibiting microglial activation on exosomes collected from the mediobasal hypothalamus. Bar graphs representing the protein levels of (a) CD13, (b) Cleaved Caspase-3 (CC3), and (c) C1q along with representative bands for AD, PF, AF, AF+Mino, AF+NTX, and AF+NTD exosome samples. The protein levels were measured by Western blot. Data are presented as mean ± SEM (n=5-6). The differences between AD, PF, AF, AF+Mino, AF+NTX, and AF+NTD were compared by one-way analysis of variance and the Newman-Keuls posttest. *, $p< 0.05$, vs AD, **, $p<0.01$, vs AD and PF, &, $p<0.05$, vs AD and PF, a, $p< 0.05$, vs AF, #, $p<0.01$, vs AF+Mino and AF+NTX.

Lastly, since postnatal ethanol exposure increased microglial specific exosomes and pro-apoptotic cargo, I determined whether these exosomes induced β-endorphin apoptosis. In order to determine this, I treated AF rats with GW4869, a neutral sphingomyelinase inhibitor that inhibits exosome formation, 15 minutes before each postnatal ethanol exposure. As previously shown, AF-treated rats had significantly less β-endorphin neurons in the arcuate nucleus compared than AD and PF controls. However, pretreatment with GW4869 completely prevented ethanol-induced loss of β-endorphin neurons (Fig. 15). Therefore, inhibition of exosome release in AF-treated rats prevents apoptosis of β-endorphin neurons.

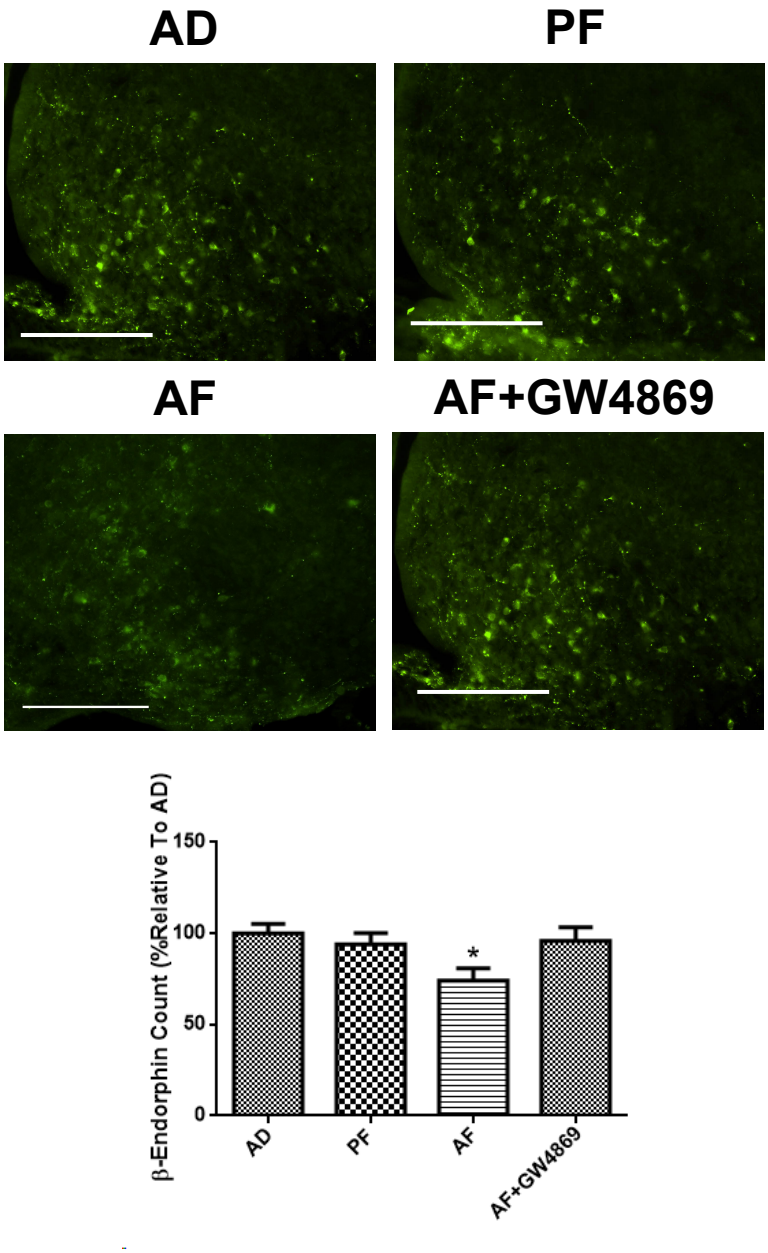


FIGURE 15. INHIBITION OF EXOSOME RELEASE (GW4869) PREVENTS ETHANOL-INDUCED LOSS OF β-ENDORPHIN NEURONS

Effect of postnatal alcohol exposure with or without GW4869 on BEP expression in the arcuate nucleus. Representative images of (a) BEP positive staining (green) along with a histogram (b) representing the mean ± SEM in AD, PF, AF, and AF+GW4869 treated rat pups (n= 5-6). Scale bars for these images are 200 μm/each. The differences between AD, PF, AF, and AF+GW4869 were compared by one-way analysis of variance (ANOVA) and the Newman-Keuls posttest. *, $p< 0.05$, AF vs PF and AD controls.

3.4 DISCUSSION

Exosomes are small vesicles (30-150 nm in diameter) that have been shown to play a role in communication between microglia and neurons (Antonucci et al., 2012; Bahrini et al., 2015; Glebov et al., 2015; Drago et al., 2016). Furthermore, neuroinflammation or neurodegenerative diseases can alter this communication by changing the cargo of microglial exosomes to promote apoptosis in recipient neurons (Sharples et al., 2008; Chang et al., 2013; Joshi et al., 2014; Jablonski et al., 2016; Kumar et al., 2017). Lastly, ethanol exposure can also alter the cargo of exosomes to promote apoptosis in recipient cells (Momen-Hervani et al., 2015; Saha et al., 2016; Cho et al., 2017). What has not been determined is whether microglial exosomes contribute to ethanol-induced apoptosis of β-endorphin neurons. In this study I first confirmed that the vesicles I collected from the MBH were exosomes. Nanoparticle tracking analysis revealed that the average size of these exosomes was 138 nm and the mode was 86 nm. Transmission electron microscopy of these vesicles also showed that individual exosomes were around within the 30-150 nm in diameter with a typical "cup-shape" that is generally observed in transmission electron microscopy of exosomes (Wu et al., 2015). Additionally, these vesicles were not contaminated by vesicle-producing organelles, as they were negative for Golgi apparatus protein GM130 and endoplasmic reticulum protein calnexin, nor did they contain mitochondrial cytochrome c. Therefore, these vesicles collected from the MBH of AD-, PF-, and AF-treated rats were exosomes.

Next, to determine whether postnatal ethanol exposure alters the cargo of MBH exosomes I assessed changes in exosomal protein with Western blot using the exosome marker CD63 as a control (Kavanagh et al., 2017). Since postnatal ethanol exposure

activates microglia, I probed for a microglial-specific marker CD13 (Potolicchio et al., 2015). I found that postnatal ethanol exposure increased CD13 protein expression on exosomes. Changes in CD13 protein were confirmed by augmented CD13 activity in AF-treated exosomes. These data suggest that postnatal ethanol exposure increased the amount of microglial exosomes. To further understand whether this increase in microglial exosomes would also alter the cargo of MBH exosomes, I probed for cleaved caspase-3 and C1q protein. Both of these proteins have been shown to induce apoptosis (Dedio et al., 1998; Porter & Jänicke, 1999; Ten et al., 2010). MBH exosomes from AF-treated rats exhibited significantly greater protein levels of cleaved caspase-3 and C1q compared to AD and PF controls. Next, if the augmented exosome release was due to microglial activation then these effects should be nullified following minocycline treatment. Indeed, minocycline administration normalized CD13 protein and activity, as well as attenuated cleaved caspase-3 and C1q expression from AF-treated rat MBH exosomes. Therefore, I posit that postnatal ethanol-induced microglial activation augments the release of pro-apoptotic microglial exosomes. On potential concern with these exosome results is that they contained cleaved caspase-3, suggesting the possibility that the microglia they come from are apoptotic. It should be noted that cytochrome c was not found in these exosomes, which is a small molecule that is released from mitochondria prior to activation of cleaved caspase 3 (Porter & Jänicke, 1999). Furthermore, enzymatic cleavage of pro-peptides can occur within exosomes to produce their active peptide (Bianco et al., 2005). In fact caspase 8, a caspase that cleaves procaspase-3 to release activated cleaved caspase-3, protein and mRNA have been found in mouse exosomes, suggesting the possibility that cleaved caspase-3 is at least partially generated within

exosomes (Valadi et al., 2007). Furthermore, I have presented data illustrating that microglial cell number increases following the current model of postnatal ethanol exposure I used. Thus, it is unlikely that the microglia secreting these pro-apoptotic exosomes are undergoing apoptosis. Another possible concern is that these vesicles are apoptotic bodies, which contain apoptotic proteins, but these samples were passed through a 0.2 μm strainer. Furthermore, no apoptotic bodies were observed with the transmission electron microscope while nanoparticle tracking analysis data demonstrated that the overwhelming majority of exosomes were less than 200 nm.

Opioid receptors have previously been shown to regulate ethanol-induced activation of microglia. Therefore, I determined whether opioid receptors regulated the release of microglial exosomes. I found that NTX administration reduced the quantity and activity of CD13 from AF-treated MBH exosomes. Meanwhile, NTD administration maintained the ethanol-induced increase. Since MOR antagonism attenuated the quantity of microglial exosomes I determined whether it altered the cargo of MBH exosomes. In concert with the previous findings, NTX reduced the amount of cleaved caspase-3 and C1q while NTD did not. Taken together, these data strongly suggest that MOR antagonism prevents ethanol-induced increases in microglial exosomes by inhibiting microglial activation. DOR antagonism does not prevent microglial activation and therefore does not prevent upregulation of microglial exosomes.

Lastly, to confirm whether these pro-apoptotic exosomes affected ethanol-induced β-endorphin apoptosis, I inhibited exosome production with GW4869. GW4869 is a sphingomyelinase inhibitor and has been shown to significantly reduce exosome production as well as prevent exosome-induced apoptosis for neurons in an Alzheimer's

disease model (Wang et al., 2012; Dinkins et al., 2014). GW4869 administration prevented the loss of β-endorphin neurons in the arcuate nucleus of AF-treated rats. This suggests that ethanol-induced apoptosis of β-endorphin is due, at least in part, to microglial exosomes.

In summary, the data presented above strongly suggests that postnatal ethanol exposure augments the release of microglial exosomes containing pro-apoptotic proteins. Moreover, microglial activation is necessary for ethanol-induced augmentation of microglial exosomes and pro-apoptotic proteins because minocycline and NTX administration blocked these effects. Lastly, prevention of the release of these exosomes with GW4869 prevents ethanol-induced apoptosis of β-endorphin neurons.

Chapter 4

4.1 INTRODUCTION

The complement system is a critical regulator of the immune system, peripherally and centrally. These signaling molecules are involved in phagocytosis of apoptotic cells, synaptic pruning, and regulation of inflammation (Stevens et al., 2007; Griffiths et al., 2009; Bohlson et al., 2014). This signaling cascade begins when complement peptide C1q binds to an apoptotic cell via recognition of apoptotic markers on the cell surface including phosphatidylserine or high-motility group box 1. Once bound to these "eat me" signals, the C1 complex becomes activated and cleaves downstream complement proteins, which leads to the formation of a transmembrane pore called the membrane attack complex (C5b-9) for opsinization and cell lysis (Rus et al., 2005).

This system is tightly regulated, however, the central nervous system does not express a majority of complement inhibitors, including CD59 and Factor H. Therefore, neurons are more susceptible to complement-induced phagocytosis and pruning in a neuroinflammatory state (Cahoy et al., 2008; Stephan et al., 2012). Evidence for this can be observed in Alzheimer's disease where, prior to the formation of Aβ plaques, C1q is augmented and related to increased synaptic pruning (Hong et al., 2016). Furthermore, C1q and C3 are elevated in mouse retina during the early stages of glaucoma, prior neuronal loss (Steele et al., 2006). Likewise, chronic ethanol exposure can upregulate cytochrome P450 expression and activate C1q and Bid-mediated apoptosis in adipose tissue (Sebastian et al., 2011). Moreover, C1q, C3, and C4 are required for liver injury, steatosis, and augmented proinflammatory cytokine expression following chronic ethanol exposure (McCullough et al., 2018). Therefore, neuroinflammatory diseases and chronic

ethanol exposure can upregulate C1q and complement signaling to dysregulate homeostasis and induce cellular apoptosis and synaptic pruning.

Microglia are the mediators of neuroinflammation and the primary source of C1q in the central nervous system (Fonseca et al., 2017). Furthermore, activation of microglia can increase the production of C1q by microglia (Silverman et al., 2016). Since I have presented data demonstrating that postnatal ethanol exposure activates microglia, I determined whether postnatal ethanol exposure also increased C1q expression in the arcuate nucleus of the hypothalamus where β-endorphin neurons are located. In addition, since deposition of C1q is a critical first step in complement-induced apoptosis of cells, I also quantified co-localization of C1q on β-endorphin neurons. Lastly, since opioid receptors can regulate microglial activation I investigated whether MOR and DOR antagonism regulate complement signaling in postnatally ethanol-exposed rats.

4.2 MATERIALS AND METHODS

Animal Use

Animal care and treatment were performed in accordance with institutional guidelines and complied with the National Institutes of Health policy and Rutgers Animal Care and Facilities Committee. Adult Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). Rats were kept under 12 hour light/12 hour dark conditions, provided water and rodent chow ad libitum, and mated. Neonates were used for postnatal ethanol exposure experiments.

Postnatal Ethanol Exposure Model

Postnatal rat pups (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 isocaloric control (PF), or they were left in the litter with their mother (AD). Gavage feeding was conducted at 10:00 AM and 12:00 PM from PND 2-6. After feeding, these pups were immediately returned to the litter. Additionally, some animals were treated subcutaneously with minocycline (45μg/kg; one hour prior to the first feeding), or naltrexone (NTX, 10mg/kg; 15 minutes prior to the first feeding), or naltrindole (NTD, 10mg/kg; 15 minutes prior to the first feeding). All of these drugs were purchased from Sigma Aldrich (St. Louis, MO). Two hours after the last feeding on PND 6, pups were transcardially perfused with 4% paraformaldehyde, postfixed overnight, cryoprotected in 30% sucrose, and cut into 30 μm coronal sections for immunohistochemistry.

Immunohistochemistry

Serial coronal sections of the 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, one PF, and one AF brain section. The sections were washed in PBS twice followed by antigen retrieval in citrate buffer (pH 6.2) at 100°C for 10 min. After two washes in PBS-T (0.05% Triton-X) the sections were incubated in blocking buffer (2.5% normal horse serum in PBS-T) at room temperature for 30 min. The sections were subsequently incubated overnight at room temperature with primary antibodies. The primary antibodies for immunohistochemistry used were rabbit anti-ß-endorphin (1:500, Peninsula Laboratories, San Carlos, CA) and mouse anti-C1q (1:500; Abcam, Cambridge, MA). After the primary antibody incubation the samples were washed in PBS and then sections were Alexa Fluor secondary antibodies (Thermo Fisher Scientific, Grand Island, NY) for immunofluorescence. Sections were then mounted with DAPI (Vector Laboratories, Burlingame, CA) and sealed with nail polish. To evaluate the immunohistochemical staining intensity, sections from each experimental group were photographed using Nikon-TE 2000 inverted microscope (Nikon Instruments Inc., Melville, NY). Pixel density and cell counting were quantified using the Fiji version ImageJ software (National Institutes of Health, Bethesda, MD). For co-localization analysis of C1q and β-endorphin neurons, the *coloc2* plugin was used, which calculates the Pearson coefficient co-localization parameter.

Statistical Analysis

Statistical analyses were conducted using Graph Pad Prism 6.0 software for Mac (Graph Pad Software, Inc., San Diego, CA). Treatment groups were compared by one-

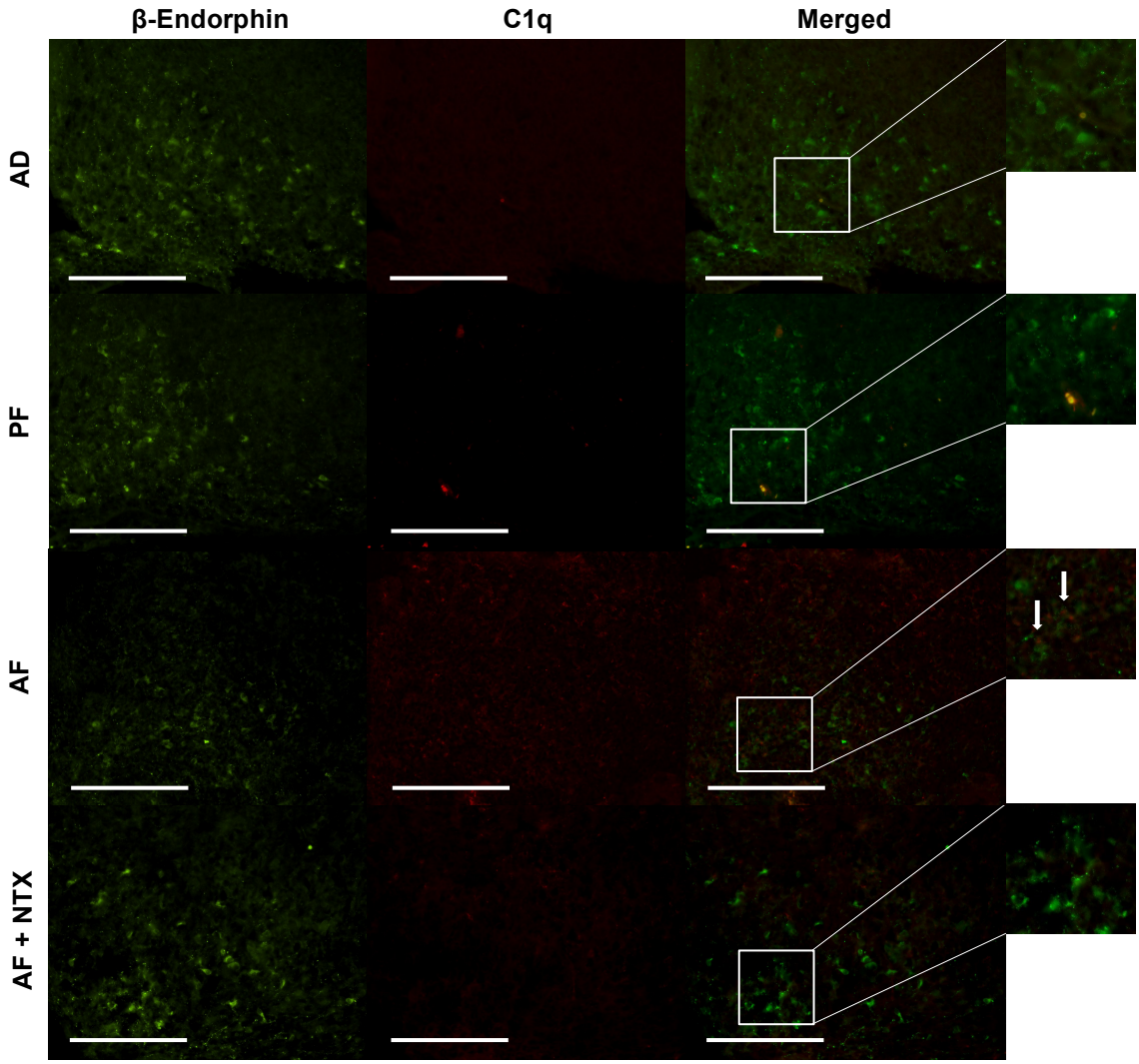
way ANOVAs with Newman-Kuels post-hoc tests. A value of $P<0.05$ was considered significant.

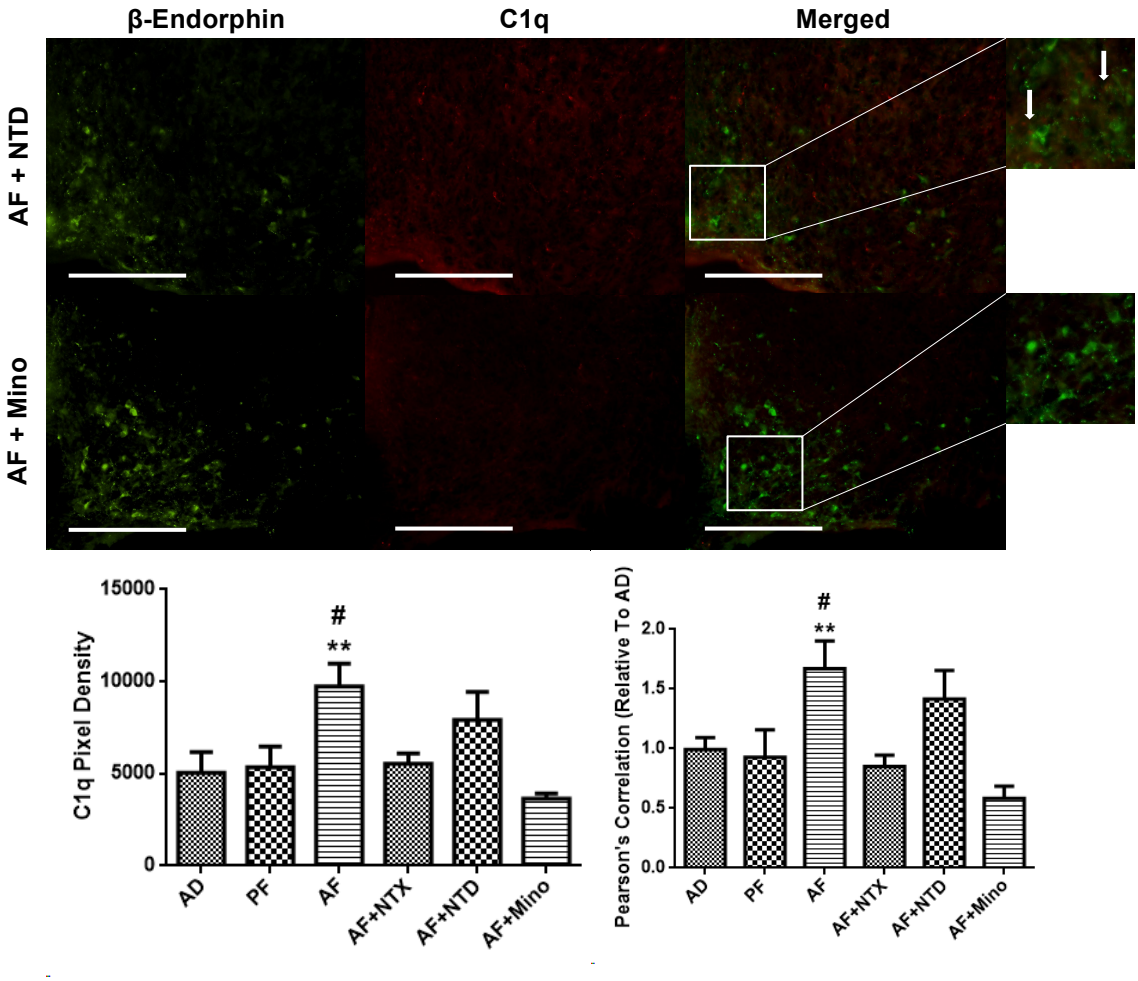
4.3 RESULTS

Complement C1q plays an important role in regulating apoptosis of cells. In the CNS, microglia are the dominant source of C1q and chronic neuroinflammation or ethanol exposure augments C1q release (Sebastian et al., 2011; Silverman et al., 2016; Fonseca et al., 2017; McCullough et al., 2018). Therefore, I set out to determine if postnatal ethanol exposure affected C1q expression and deposition on β-endorphin neurons in the arcuate nucleus. Immunofluorescent staining revealed that AF-treated rats exhibited significantly greater C1q expression than AD and PF controls, represented by greater fluorescence intensity (Fig. 16). Furthermore, Pearson's correlation coefficient showed that C1q and β-endorphin co-localization was greater in AF-treated rats. Since microglial activation can augment C1q expression, I determined whether ethanol-induced microglial activation was involved in increased C1q staining and co-localization in AF-treated rats by inhibiting microglial activation with minocycline. Inhibition of microglial activation completely prevented ethanol-induced increases in C1q expression and co-localization on β-endorphin neurons. Therefore, postnatal ethanol exposure activates microglia to produce C1q that then deposits on β-endorphin neurons. Next, since I have previously shown that opioid receptors regulate microglial activation, I determined whether MOR or DOR antagonism would regulate C1q expression and co-localization on β-endorphin neurons. NTX significantly reduced C1q expression and co-localization in AF-treated rats. NTD did not prevent the ethanol-induced effect, as the data for AF+NTD were no different than AF in either assessment. Therefore, MOR antagonism may reduce C1q expression and co-localization through inhibition of ethanol-induced microglial activation.

FIGURE 16. REGULATION OF POSTNATAL ETHANOL EXPOSURE INDUCED CHANGES IN C1q EXPRESSION AND CO-LOCALIZATION ON β-ENDORPHIN NEURONS IN THE ARCUATE NUCLEUS

The effects of postnatal ethanol exposure on C1q expression and co-localization on β-endorphin neurons in the arcuate nucleus, as well as minocycline, NTX, and NTD administration. Representative images of C1q positive staining (red) and BEP positive staining (green), along with merged images and a zoomed in picture to demonstrate co-localization. Scale bars for these images are 200 μm/each. Histograms representing the mean ± SEM in AD, PF, AF, AF+NTX, AF+NTD, and AF+Mino rat pups on PND 6 (n=5-8). The differences between treatment groups were compared by one-way analysis of variance (ANOVA) and the Newman-Keuls posttest. **, $p < 0.01$, AF vs AF+Mino, #, $p<0.05$, AF vs AD, PF, and AF+NTX.


β-Endorphin
C1q
Merged
AD
PF
AF
AF + NTX


β-Endorphin
C1q
Merged
AF + NTD
AF + Mino
C1q Pixel Density
15000
10000
5000
0
#
**
AD
PF
AF
AF+NTX
AF+NTD
AF+Mino
Pearson's Correlation (Relative To AD)
2.0
1.5
1.0
0.5
0.0
#
**
AD
PF
AF
AF+NTX
AF+NTD
AF+Mino

4.4 DISCUSSION

Postnatal ethanol exposure induces apoptosis of β-endorphin neurons, however, the role complement proteins have in this has yet to be explored. Here I have presented data that suggests complement proteins, focusing on C1q, are involved. First, ethanol exposure has been shown to upregulate C1q and increase apoptosis in the liver and microglial activation leads to an augmentation of C1q release (Sebastian et al., 2011; McCullough et al., 2018). Therefore, I examined the expression of C1q in the arcuate nucleus. AF-treatment significantly increased the expression of C1q in the arcuate nucleus relative to AD and PF controls. However, C1q must be deposited on neurons in order for the classical complement pathway to begin and produce the membrane attack complex to lyse cells. Therefore, I determined whether C1q co-localized with β-endorphin neurons in the arcuate nucleus utilizing Pearson's correlation coefficient. Pearson's correlation coefficient was used because it subtracts the mean intensity from each pixel's intensity value and is independent of signal levels and background. This allows Pearson's correlation coefficient to be independent of any form of preprocessing and relatively safe from user bias (Dunn et al., 2011). Pearson's correlation coefficient determined that C1q and β-endorphin signals were co-localized significantly more in AF-treated rats than AD and PF controls. Since Fonseca et al. demonstrated that microglia are the dominant producer of C1q, I determined whether the increase in C1q expression was due to ethanol-induced microglial activation (Fonseca et al., 2017). Therefore, inhibition of microglial activation with minocycline normalized C1q fluorescence intensity and co-localization in AF-treated rats. These data show that postnatal ethanol exposure activates microglia to produce C1q, which gets deposited on β-endorphin neurons.

Deposition of C1q is critical to complement mediated apoptosis, as activation of the C1 complex is required to generate the membrane attack complex. First, the C1 complex cleaves complement components C2 and C4. Their cleavage products, C2a and C4b, bind together to form the C3 convertase and adhere to the cell. C3 convertase can then cleave C3 into C3a and C3b, C3b binds to the C3 convertase to form the C5 convertase, and the C5 convertase cleaves C5, which leads to the formation of the membrane attack complex and cell lysis (Rus et al., 2005). Another reason why C1q deposition is important is because Cd11b (also known as complement C3 receptors) on microglia recognizes and binds to C3b to induce phagocytosis of neurons (Arcuri et al., 2017). Therefore, C1q deposition is required for complement induced phagocytosis and cell lysis of β-endorphin neurons.

Since my previous data has shown that opioid receptors regulated microglial activation, I determined whether MOR and DOR antagonism regulated ethanol-induced increases in C1q expression and co-localization on β-endorphin neurons. Consistent with previous findings, NTX significantly reduced C1q staining in the arcuate nucleus and co-localization on β-endorphin neurons. This is further evidence that ethanol-induced changes in the complement system are due to activation of microglia since my previous data has demonstrated that MOR antagonism prevents ethanol-induced microglial activation. Another potential mechanism involved in the protective effects of MOR antagonism could be the downregulation of MOR and upregulation of DOR (Boyadjieva et al., 2004; Sarkar et al., 2012). DOR has been suggested to play a role in preventing ethanol-induced microglial activation and promoting an anti-inflammatory environment to protect neurons and maintain neuronal homeostasis (Shrivastava et al., 2017).

Likewise, NTD treatment did not inhibit the ethanol effect as AF+NTD treated rats had no difference in C1q expression or co-localization on β-endorphin neurons compared to AF rats. Furthermore, DOR antagonism may downregulate DOR and upregulate MOR, which can prevent anti-inflammatory processes and aid in ethanol-induced increases in C1q expression and co-localization on β-endorphin neurons (Sarkar et al., 2012).

In summary, postnatal ethanol exposure activates microglia to augment production of C1q. This increased production of C1q also corresponds with increased deposition on β-endorphin neurons and may play a role in ethanol-induced apoptosis of these neurons. Since MOR regulates ethanol-induced microglial activation, antagonism of MOR completely prevented ethanol-induced increases in C1q expression and co-localization on β-endorphin neurons. DOR antagonism had no effect on the ethanol-induced increases in C1q expression and co-localization

SUMMARY AND CONCLUSIONS

There are 4 conclusions that can be taken from this dissertation: 1) Postnatal ethanol exposure activates microglia in the arcuate nucleus to reduce the number of β-endorphin neurons through induction of the M1-phenotype, upregulation of proinflammatory cytokines, and promoting a phagocytic-like phenotype. 2) Exosomes released from microglia following postnatal ethanol exposure contain pro-apoptotic peptides that contribute to β-endorphin neuronal apoptosis. 3) C1q expression and deposition are upregulated in response to postnatal ethanol exposure and may contribute to ethanol-induced apoptosis of β-endorphin neurons through phagocytosis and cell lysis. 4) Opioid receptors, notably MOR are vital to ethanol-induced activation of microglia since NTX administration attenuated ethanol induced-microglial activation, microglial exosome production, C1q expression and deposition, and apoptosis of β-endorphin neurons. DOR antagonism does not affect ethanol-induced microglial activation.

Data from this dissertation confirms previous findings from Dr. Sarkar's lab that postnatal ethanol exposure induces apoptosis of β-endorphin neurons. Furthermore, I found that the reduction in β-endorphin neurons was due primarily to ethanol-induced activation of microglia. This is because activated microglia exhibited an M1-phenotype, produced more proinflammatory cytokines (TNF-α and MCP1), and altered interactions with POMC neurons, suggesting increased phagocytosis of these neurons. Examination of these microglia found augmentation in TLR-4, TLR-4 dependent signaling, which is involved in apoptotic proinflammatory signaling, and increased MOR on the surface of microglia. Increased expression of MOR suggested that opioid receptors might play a role in ethanol-induced activation of microglia considering that β-endorphin is a peptide

that can bind to MOR and DOR. Therefore, I determined whether MOR and DOR antagonism differentially regulated microglia and prevented ethanol-induced neurotoxicity. I found that MOR antagonism significantly attenuated TLR-4 signaling in microglia and prevented β-endorphin neuronal cell loss in AF-NTX-treated rats. DOR antagonism did not prevent these ethanol-induced phenotypes.

Next, exosomes have recently been shown to serve as a form of communication between cells and can even carry apoptotic cargo that induces cell death. Therefore I assayed MBH exosomes and found that ethanol-exposed rats had significantly more microglial exosomes (CD13) and pro-apoptotic proteins (C1q and cleaved caspase-3). The increased microglial exosomes and pro-apoptotic peptides were attenuated in AF rats that were co-treated with NTX or minocycline, demonstrating that microglial activation is necessary for augmented microglial exosome production and pro-apoptotic cargo. NTD did not affect this phenotype, as AF+NTD data was not significantly different from AF. Furthermore, inhibition of exosome release with GW4869 prevented the reductions of β-endorphin neurons observed in AF-treated rats. Therefore, postnatal ethanol exposure activates microglia to release exosomes carrying pro-apoptotic cargo and inhibition of their release can prevent apoptosis of β-endorphin neurons.

Lastly, complement signaling is known to regulate apoptosis and can be dyregulated to induce apoptosis following chronic ethanol exposure. Therefore I determined whether postnatal ethanol exposure altered C1q signaling the arcuate nucleus. I found that AF-treated rats exhibited significantly greater C1q expression, as well as C1q co-localization with β-endorphin neurons. The latter point is critical because deposition of C1q on cells is the first step for complement-induced phagocytosis and cell lysis.

Inhibition ethanol-induced activation of microglia with NTX or minocycline normalized C1q expression and β-endorphin co-localization. These suggested that ethanol-induced activation of microglia upregulated C1q expression and deposition on β-endorphin neurons.

In conclusion, postnatal ethanol exposure induces apoptosis of β-endorphin neurons primarily through microglial activation (Fig. 16). Activation of microglia has been shown to produce proinflammatory cytokines (TNF-α & MCP1), induce a phagocytic appearance when interacting with POMC neurons, augment TLR-4 and downstream signaling molecules (p38 MAPK, p-Akt, p-JNK, and NFκB) that induce proinflammatory production, augment pro-apoptotic microglial exosomes, and C1q expression and deposition on β-endorphin. Taken together all of these factors contribute to ethanol-induced apoptosis of β-endorphin neurons. Minocycline, a potent inhibitor of microglial activation, and NTX administration attenuated microglial activation and completely blocked all of ethanol's effects. These data suggest that NTX and minocycline may have to potential to prevent some of the deleterious effects of fetal alcohol exposure; however, more research is needed to confirm this possibility.

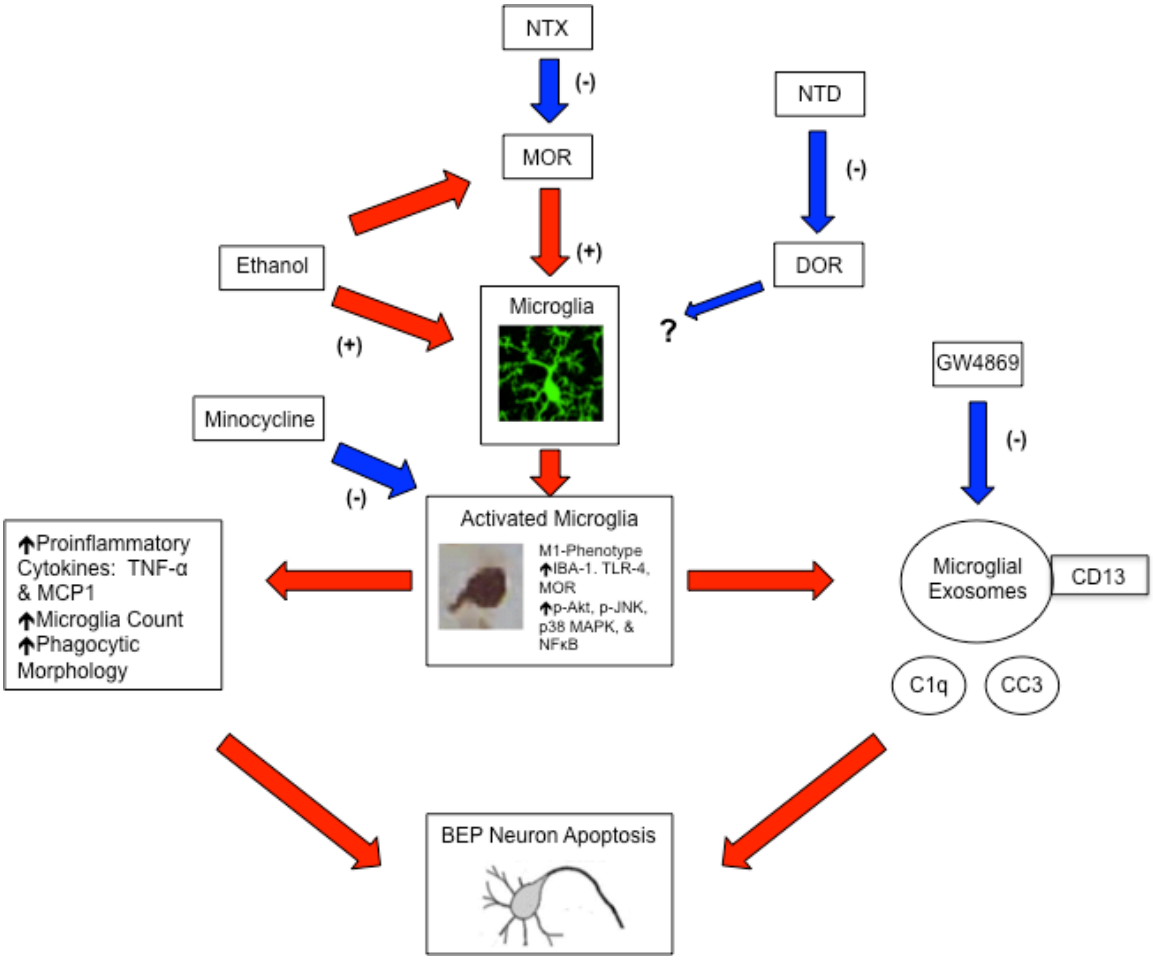
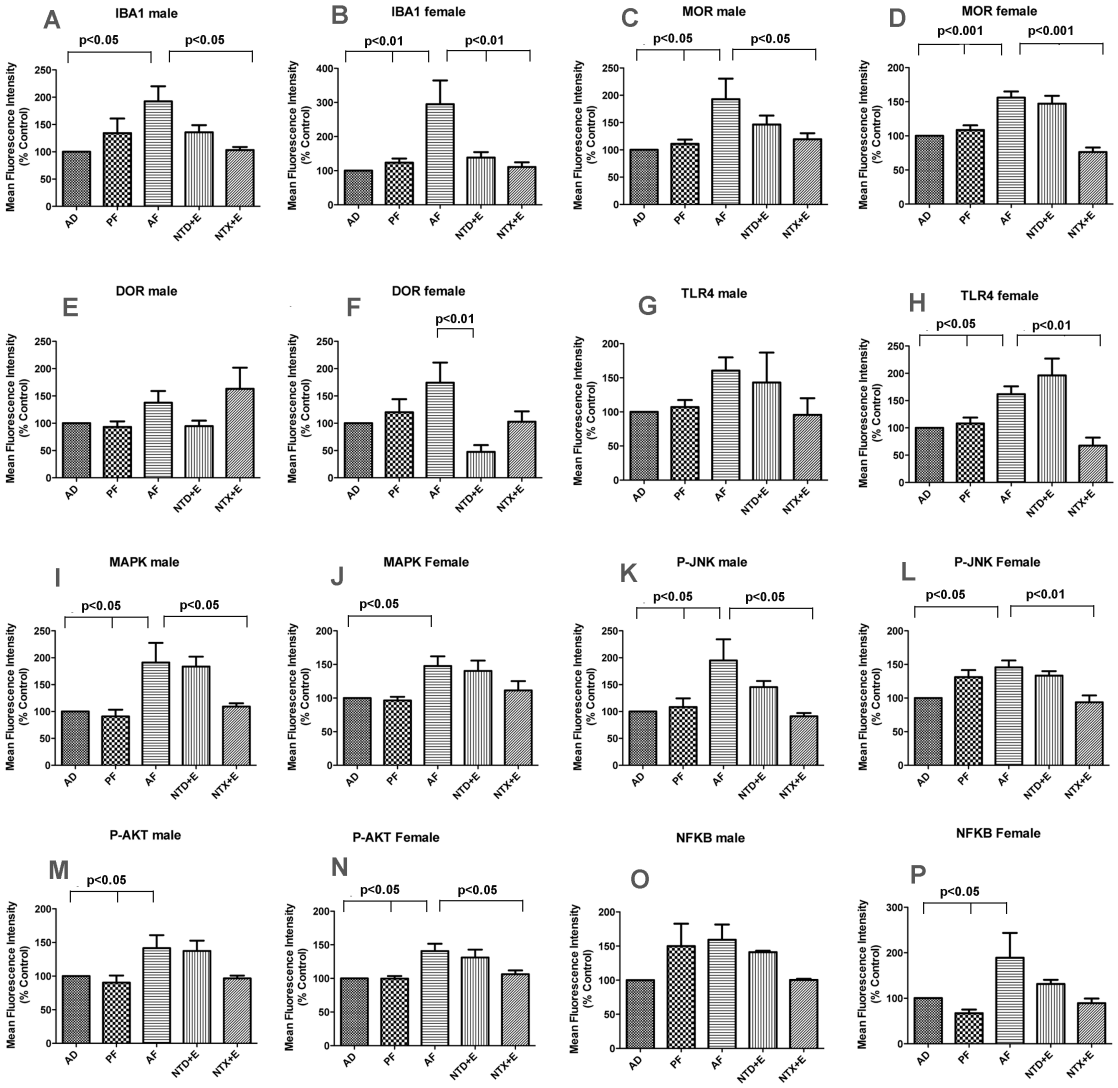


FIGURE 17. PROPOSED MECHANISM FOR POSTNATAL ETHANOL-INDUCED APOPTOSIS OF β-ENDORPHIN NEURONS

Schematic diagram illustrating the proposed mechanism by which ethanol interacts with opioid receptors to control β-endorphin neuronal apoptosis in the hypothalamus. Ethanol activates microglial to produce proinflammatory cytokines, proliferate, and adopt phagocytic morphology. Chronic inflammation and overproduction of TNF-α are cytotoxic to β-endorphin neurons. Additionally, activated microglia produce exosomes with pro-apoptotic proteins cleaved caspase-3 and C1q that can contribute to β-endorphin neuronal apoptosis. This effect of exosomes was blocked by GW4869. Inhibition of microglial activation with minocycline completely attenuated the ethanol-induced effect. MOR antagonist inhibited ethanol-induced apoptosis of β-endorphin neurons, as well at the ethanol phenotype suggesting that MOR activation is critical for ethanol-induced activation of microglia. DOR antagonism did not affect this model.

Appendix



APPENDIX FIGURE 1. POSTNATAL ETHANOL EFFECT ON MICROGLIAL OPIOID RECEPTORS AND TLR4 PATHWAY PROTEINS IN THE MEDIOBASAL HYPOTHALAMUS OF MALE AND FEMALE NEONATES.

Sex differences in protein quantification from isolated microglia were not evident in AD, PF, AF, AF+NTX, and AF+NTD rat pups at PND 6 by flow cytometry. Bar graphs show mean or median fluorescence intensity of staining of IBA-1 (A,B), MOR (C,D), DOR (E,F), TLR4 (G,H), p-38 MAPK (I,J), p-JNK (K,L), p-AKT (M,N), and NF-KB (0,P) from microglia isolated from each treatment group from male and female neonates. Data are represented as Mean ± SEM (n= 3-7) and were compared by one-way analysis of variance and Newman-Keuls posttest. Differences between groups are shown by lines with *p* values on the top of bar graphs.

Acknowledgment of Previous Publications

Most of the information obtained from the studies comprising chapter two of this dissertation has been recently published:

Shrivastava, P*., **Cabrera, M. A***., Chastain, L. G., Boyadjieva, N. I., Jabbar, S., Franklin, T., & Sarkar, D. K. (2017). Mu-opioid receptor and delta-opioid receptor differentially regulate microglial inflammatory response to control proopiomelanocortin neuronal apoptosis in the hypothalamus: effects of neonatal alcohol. Journal Of Neuroinflammation, 14(1):83-102. doi:10.1186/s12974-017-0844-3

* Connotes co-author

References

1. Abrami L, Brandi L, Moayeri M, Brown MJ, Krantz BA, Leppla, S.H., & van der Goot, F.G. (2013). Hijacking multivesicular bodies enables long-term and exosome-mediated long-distance action of anthrax toxin. *Cell Reports, 5*(4):986–996

2. Agapito, M. A., Barreira, J. C., Logan, R. W., & Sarkar, D. K. (2013). Evidence for possible Period2 gene mediation of the effects of alcohol exposure during the postnatal period on genes associated with maintaining metabolic signaling in the mouse hypothalamus. *Alcoholism, Clinical and Experimental Research, 37*(2):263–269. http://doi.org/10.1111/j.1530-0277.2012.01871.x

3. Aguilera, G., & Liu, Y. (2012). The Molecular Physiology Of CRH Neurons. *Frontiers in Neuroendocrinology, 33*(1):67–84. http://doi.org/10.1016/j.yfrne.2011.08.002

4. Akhter, N., Nix, M., Abdul, Y., Singh, S., & Husain, S. (2013). Delta-Opioid Receptors Attenuate TNF-α–Induced MMP-2 Secretion From Human ONH Astrocytes. *Investigative Ophthalmology & Visual Science, 54*(10):6605–6611. http://doi.org/10.1167/iovs.13-12196

5. Alikunju, S., Abdul Muneer, P. M., Zhang, Y., Szlachetka, A. M., & Haorah, J. (2011). The inflammatory footprints of alcohol-induced oxidative damage in neurovascular components. *Brain Behavior & Immunity, 25*(Suppl. 1):S129–S136.

6. Alliot, F., Godin, I., & Pessac, B. (1999). Microglia derive from progenitors, originating from the yolk sac, and which proliferate in the brain. Developmental Brain Research, 117(2), 145-152.

7. Anna, G., Tamar, S., Trent M., W., & Orly, R. (2017). Serping1/C1 Inhibitor Affects Cortical Development in a Cell Autonomous and Non-cell Autonomous Manner. *Frontiers In Cellular Neuroscience, 11*(169):1-14 doi:10.3389/fncel.2017.00169/full

8. Antonucci, F., Turola, E., Riganti, L., Caleo, M., Gabrielli, M., Perrotta, C., Novellino, L., Clementi, E., Giussani, P., Viani, P., Matteoli, M., & Verderio, C. (2012). Microvesicles released from microglia stimulate synaptic activity via enhanced sphingolipid metabolism. *EMBO Journal, 31*(5):1231-1240

9. Arcuri, C., Mecca, C., Bianchi, R., Giambanco, I., & Donato, R. (2017). The Pathophysiological Role of Microglia in Dynamic Surveillance, Phagocytosis and Structural Remodeling of the Developing CNS. *Frontiers in Molecular Neuroscience, 10*:191. http://doi.org/10.3389/fnmol.2017.00191

10. Bahrini, I., Song, J., Hanayama, R., & Diez, D. (2015). Neuronal exosomes facilitate synaptic pruning by up-regulating complement factors in microglia. *Scientific Reports, 5*:7989. doi:10.1038/srep07989

11. Bai, D., Ueno, L., & Vogt, P. K. (2009). Akt-mediated regulation of NFκB and the essentialness of NFκB for the oncogenicity of PI3K and Akt. International Journal of Cancer. *Journal International Ductal Cancer*, *125*(12):2863–2870. http://doi.org/10.1002/ijc.24748

12. Bao, W. L., Williams, A. J., Faden, A. I., & Tortella, F. C. (2001). Selective mGluR5 receptor antagonist or agonist provides neuroprotection in a rat model of focal cerebral ischemia. *Brain Research, 922*(2):173–9.

13. Barres, C., Blanc, L., Bette-Bobillo, P., Andre, S., Mamoun, R., Gabius, H.J., & Vidal, M. (2010). Galectin-5 is bound onto the surface of rat reticulocyte exosomes and modulates vesicle uptake by macrophages. *Blood, 115*(3), 696-705

14. Beattie, M. S. (2004). Inflammation and apoptosis: linked therapeutic targets in spinal cord injury. *Trends in Molecular Medicine, 10*(12):580–583.

15. Bekdash, R. A., Zhang, C., & Sarkar, D. K. (2013). Gestational choline supplementation normalized fetal alcohol-induced alterations in histone modifications, DNA methylation and POMC gene expression in β-endorphin-producing POMC neurons of the hypothalamus. *Alcoholism, Clinical and Experimental Research, 37*(7), 1133–1142. http://doi.org/10.1111/acer.12082

16. Bell-Temin, H., Zhang, P., Chaput, D., King, M. A., You, M., Liu, B., & Stevens, S. M., Jr. (2013). Quantitative proteomic characterization of ethanol-responsive pathways in rat microglial cells. *Journal of Proteome Research, 12*(5):2067-2077. doi: 10.1021/pr301038f

17. Bertagna, X. (1994). Proopiomelanocortin-derived peptides. *Endocrinology and Metabolism Clinics of North America, 23*(3):467-85.

18. Bertrand J., Floyd R.L., Weber M.K., O'Connor M., Riley E.P., Johnson K.A., & Cohen D.E. (2004). National Task Force on FAS/FAE. Fetal Alcohol Syndrome: Guidelines for Referral and Diagnosis. Atlanta, GA: Centers for Disease Control and Prevention.

19. Bialas, A. R., & Stevens, B. (2013). TGF-β signaling regulates neuronal C1q expression and developmental synaptic refinement. *Nature Neuroscience, 16*(12):1773-1782. doi:10.1038/nn.3560

20. Bianco, F., Pravettoni, E., Colombo, A., Schenk, U., Möller, T., Matteoli, M., & Verderio, C. (2005). Astrocyte-Derived ATP Induces Vesicle Shedding and IL-1β Release from Microglia. *Journal of Immunology, 174*(11):7268-7277; DOI: https://doi.org/10.4049/jimmunol.174.11.7268

21. Bicknell, A.B., The tissue-specific processing of pro-opiomelanocortin. Journal of *Neuroendocrinology, 20*(6):692-9.

22. Bilbo, S. D. & Schwarz, J. M. (2012). The immune system and developmental programming of brain and behavior. *Frontiers of Neuroendocrinology, 33*:267–86.

23. Blanco, A., Valles, S., Pascual, M., & Guerri, C. (2005). Involvement of TLR4/type IIL-1 receptor signaling in the induction of inflammatory mediators and cell death induced by ethanol in cultured astrocytes. *Journal Of Immunology, 175*(10):6893-6899.

24. Block, M. L. (2008). NADPH oxidase as a therapeutic target in Alzheimer's disease. *BMC Neuroscience, 9*(S2):S8.

25. Bohlson, S., O'Conner, S., Hulsebus, H., Ho, M., & Fraser, D. (2014). Complement, C1q, and C1q-related molecules regulate macrophage polarization. *Frontiers In Immunology, 5*(402). doi: 10.3389/fimmu.2014.00402

26. Boyadjieva, N., Dokur, M., Advis, J. P., Meadows, G. G., & Sarkar, D. K. (2001). Chronic ethanol inhibits NK cell cytolytic activity: role of opioid peptide beta-endorphin. *Journal of Immunology, 167*(10):5645-5652.

27. Boyadjieva, N., Chaturvedi, K., Poplawski, M. M., & Sarkar, D. K. (2004). Opioid antagonist naltrexone disrupts feedback interaction between mu and delta opioid receptors in splenocytes to prevent alcohol inhibition of NK cell function. *Journal of Immunology, 173*(1):42-49.

28. Boyadjieva, N., & Sarkar, D. (2010). Role of Microglia in Ethanol's Apoptotic Action on Hypothalamic Neuronal Cells in Primary Cultures. *Alcoholism-Clinical And Experimental Research, 34*(11):1835-1842.

29. Boyadjieva, N., & Sarkar, D. (2013). Microglia Play a Role in Ethanol-Induced Oxidative Stress and Apoptosis in Developing Hypothalamic Neurons. *Alcoholism: Clinical And Experimental Research, 37*(2):252-262. doi:10.1111/j.1530-0277.2012.01889.x

30. Brandon-Warner, E., Feilen, N., Culberson, C. R., Field, C. O., deLemos, A. S., Russo, M. W., & Schrum, L. W. (2016). Processing of miR17-92 Cluster in Hepatic Stellate Cells Promotes Hepatic Fibrogenesis During Alcohol-Induced Injury. *Alcoholism, Clinical and Experimental Research, 40*(7):1430–1442. http://doi.org/10.1111/acer.13116

31. Budnik, V., Ruiz-Cañada, C., & Wendler, F. (2016). Extracellular vesicles round off communication in the nervous system. *Nature Reviews Neuroscience, 17*(3):160-172. doi:10.1038/nrn.2015.29

32. Burd, L., Blair, J., & Dropps, K. (2012) Prenatal alcohol exposure, blood alcohol concentrations and alcohol elimination rates for the mother, fetus and newborn. *Journal of Perinatology, 32*(9):652–659

33. Bykov, I., Junnikkala, S., Pekna, M., Lindros, K. O., & Meri, S. (2007). Effect of chronic ethanol consumption on the expression of complement components and acute-phase proteins in liver. *Clinical Immunology, 124*(2), 213-220. doi:10.1016/j.clim.2007.05.008

34. Cahoy, J.D., Emery, B., Kaushal, A., Foo, L.C., & Zamanian, J.L. (2008). A transcriptome database for astrocytes, neurons, and oligodendrocytes: a new resource for understanding brain development and function. *Journal of Neuroscience, 28*(1), 264–278.

35. Cai, Y., Teo, B., Yeo, J., & Lu, J. (2015). C1q protein binds to the apoptotic nucleolus and causes C1 protease degradation of nucleolar proteins. *Journal Of Biological Chemistry, 290*(37):22570-22580. doi:10.1074/jbc.M115.670661

36. Callaghan, D. A., Dong, L., Callaghan, S. M., Hou, Y. X., Dadmino, L., & Slack, R. S. (1999). Neural precursor cells differentiating in the absence of Rb exhibit delayed terminal mitosis and deregulated E2F 1 and 3 activity. *Developmental Biology, 207*:257– 270.

37. Centers for Disease Control and Prevention. (2000). Alcohol consumption among pregnant and childbearing-aged women-United States 1991-1999. MMWR: Morbidity & Mortality Weekly Report, 51(13), 273-276.

38. Centers for Disease Control and Prevention. (2015). Fetal Alcohol Syndrome Among Children Aged 7-9 Years – Arizona, Colorado, and New York, 2010. MMWR: Morbidity & Mortality Weekly Report, 64(3), 54-57.

39. Centers for Disease Control and Prevention, National Center for Chronic Disease Prevention and Health Promotion, Division of Population Health. BRFSS Prevalence & Trends Data [online]. 2015.

40. Centers for Disease Control and Prevention. (2018). Fetal Alcohol Spectrum https://www.cdc.gov/ncbddd/fasd/facts.html

41. Chan, H. C. S., McCarthy, D., Li, J., Palczewski, K., & Yuan, S. (2017). Designing Safer Analgesics via μ-Opioid Receptor Pathways. *Trends in Pharmacological Sciences, 38*(11):1016–1037. http://doi.org/10.1016/j.tips.2017.08.004

42. Chang C, Lang H, Geng N, Wang J, Li N, Wang X. (2013). Exosomes of BV-2 cells induced by alpha-synuclein: important mediator of neurodegeneration in PD. *Neuroscience Letters, 548*:190–195. doi: 10.1016/j.neulet.2013.06.009.

43. Chastain LG, Sarkar DK. (2014). Role of microglia in regulation of ethanol neurotoxic action. *International Review of Neurobiology, 118*:81–103.

44. Chandrasekar, B., Mummidi, S., Perla, R. P., Bysani, S., Dulin, N. O., Liu, F., & Melby, P. C. (2003). Fractalkine (CX3CL1) stimulated by nuclear factor kappaB (NF-kappaB)-dependent inflammatory signals induces aortic smooth muscle cell proliferation through an autocrine pathway. *Biochemical Journal*, *373*(Pt 2):547–558. http://doi.org/10.1042/BJ20030207

45. Chen, C., Kuhn, P., Advis, J., & Sarkar, D. (2006a). Prenatal ethanol exposure alters the expression of period genes governing the circadian function of beta-endorphin neurons in the hypothalamus. *Journal Of Neurochemistry, 97*(4):1026-1033

46. Chen, C., P., Kuhn, P., Chaturvedi, K., Boyadjieva, N., & Sarkar, D. K. (2006b). Ethanol induces apoptotic death of developing beta-endorphin neurons via suppression of cyclic adenosine monophosphate production and activation of transforming growth factor-beta1-linked apoptotic signaling. *Molecular Pharmacology, 69*(3):706-717. doi: 10.1124/mol.105.017004

47. Chen, W., Kelly, M.A., Opitz-Araya, X., Thomas, R.E., Low, M.J., & Cone, R.D. (1997) Exocrine gland dysfunction in MC5-R-deficient mice: evidence for coordinated regulation of exocrine function by melanocortin peptides. *Cell, 91*:789–798.

48. Chen, Z., Su, Y., Zhang, H., Gu, P., & Gao, L. (2014). The role of the globular heads of the C1q receptor in HPV-16 E2-induced human cervical squamous carcinoma cell apoptosis via a mitochondria-dependent pathway. *Journal Of Translational Medicine, 12*(1). doi:10.1186/s12967-014-0286-y\

49. Cherry, J. D., Olschowka, J. A., & O'Banion, M. K. (2014). Are "Resting" Microglia More "M2"? *Frontiers in Immunology, 5*:594. http://doi.org/10.3389/fimmu.2014.00594

50. Chhor, V., Le Charpentier, T., Lebon, S., Ore, M. V., Celador, I. L., Josserand, J., Degos, V., Jacotot, E., Hagberg, H., Savman, K., Mallard, C., Gressens, P., & Fleiss, B. (2013). Characterization of phenotype markers and neuronotoxic potential of polarised primary microglia in vitro. *Brain Behavior & Immunity, 32*:70-85. doi: 10.1016/j.bbi.2013.02.005

51. Chivet, M., Javalet, C., Laulagnier, K., Blot, B., Hemming, F. J., & Sadoul, R. (2014). Exosomes secreted by cortical neurons upon glutamatergic synapse activation specifically interact with neurons. *Journal of Extracellular Vesicles, 3*(0):1-10 10.3402/jev.v3.24722. http://doi.org/10.3402/jev.v3.24722

52. Cho, Y.-E., Mezey, E., Hardwick, J. P., Salem, N., Clemens, D. L., & Song, B.-J. (2017). Increased ethanol-inducible cytochrome P450-2E1 and cytochrome P450 isoforms in exosomes of alcohol-exposed rodents and patients with alcoholism

through oxidative and endoplasmic reticulum stress. *Hepatology Communications*, *1*(7):675–690. http://doi.org/10.1002/hep4.1066

53. Choi, S. H., Aid, S., Kim, H. W., Jackson, S. H., & Bosetti, F. (2012). Inhibition of NADPH oxidase promotes alternative and anti-inflammatory microglial activation during neuroinflammation. Journal of Neurochemistry, 120:292–301.

54. Circu, M. L., & Aw, T. Y. (2010). Reactive oxygen species, cellular redox systems and apoptosis. *Free Radical Biology & Medicine, 48*(6):749–762. http://doi.org/10.1016/j.freeradbiomed.2009.12.022

55. Clark, A.J. (2016). 60 YEARS OF POMC: The proopiomelanocortin gene: discovery, deletion and disease. *Journal of Molecular Endocrinology, 56*(4):T27-37. doi: 10.1530/JME-15-0268.

56. Clark, A.J., McLoughlin, L., & Grossman, A. (1993). Familial glucocorticoid deficiency associated with point mutation in the adrenocorticotropin receptor. *Lancet, 341*:461–462.

57. Cohen, J., Roychowdhury, S., Mcmullen, M., Stavitsky, A., & Nagy, L. (2009). Complement and Alcoholic Liver Disease: Role of C1q in the Pathogenesis of Ethanol-Induced Liver Injury in Mice. *Gastroenterology, 139*(2), 664-674.

58. Coleman, L. G., Zou, J., & Crews, F. T. (2017). Microglial-derived miRNA let-7 and HMGB1 contribute to ethanol-induced neurotoxicity via TLR7. *Journal of Neuroinflammation, 14*(22):1-15. http://doi.org/10.1186/s12974-017-0799-4

59. Coles, C. (1994). Critical periods for prenatal alcohol exposure. *Alcohol Health & Research World, 18*(1):22-22.

60. Colombo, M., Raposo, G., & Théry, C. (2014). Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. *Annual Review Of Cell & Developmental Biology, 30*(1), 255-289. doi:10.1146/annurev-cellbio-101512-122326

61. Cone, J. B. (2001). Inflammation. *American Journal of Surgery, 182*:558–562

62. Cone, R.D. (2005). Anatomy and regulation of the central melanocortin system. *Nature Neuroscience Review, 8*(5):571-578.

63. Contet, C., Kim, A., Le, D., Iyengar, S., Kotzebue, R. W., Yuan, C. J., Kieffer, B. L., & Mandyam, C. D. (2014). Mu Opioid Receptors Mediate the Effects of Chronic Ethanol Binge Drinking on the Hippocampal Neurogenic Niche. *Addiction Biology, 19*(5):770–780. http://doi.org/10.1111/adb.12040

64. Cool, D.R., Normant, E., Shen, F., Chen, H., Pannell, L., Zhang, Y., & Loh, Y.P. 1997 Carboxypeptidase E is a regulated secretory pathway sorting receptor: genetic obliteration leads to endocrine disorders in Cpefat mice. *Cell, 88*:73–83.

65. Cunningham, C. L., Martínez-Cerdeño, V., & Noctor, S. C. (2013). Microglia regulate the number of neural precursor cells in the developing cerebral cortex. *The Journal of Neuroscience, 33*(10):4216–4233. http://doi.org/10.1523/JNEUROSCI.3441-12.2013

66. D'Agostino, G., & Diano, S. (2010). Alpha-Melanocyte stimulating hormone: production and degradation. *Journal of Molecular Medicine (Berlin, Germany), 88*(12):1195–1201. http://doi.org/10.1007/s00109-010-0651-0

67. Dahl M.R., Thiel S., Matsushita M. (2001) MASP-3 and its association with distinct complexes of the mannan-binding lectin complement activation pathway. *Immunity, 15*(1):127–135.

68. Day, M. L., Foster, R. G., Day, K. C., Zhao, X., Humphrey, P., Swanson, P., Postigo, A. A., Zhang, S. H., & Dean, D. C. (1997). Cell anchorage regulates apoptosis through the retinoblastoma tumor suppressor/E2F pathway. *Journal of Biological Chemistry, 272*:8125–8128.

69. Deakin, J.F., Doströvsky, J.O., & Smyth, D.G. (1980). Influence of N-terminal acetylation and C-terminal proteolysis on the analgesic activity of beta-endorphin. *Biochemical Journal, 189*(3): 501–506.

70. Dedio, J., Jahnen-Dechent, W., Bachmann, M., & Müller-Esterl, W. (1998). The multiligand-binding protein gC1qR, putative C1q receptor, is a mitochondrial protein. *Journal Of Immunology, 160*(7):3534-3542.

71. Dinkins, M. B., Dasgupta, S., Wang, G., Zhu, G., & Bieberich, E. (2014). Exosome reduction in vivo is associated with lower amyloid plaque load in the 5XFAD mouse model of Alzheimer's disease. *Neurobiology Of Aging, 35*(8):1792-1800. doi:10.1016/j.neurobiolaging.2014.02.012

72. Dinkins, M., Enasko, J., Wang, G., Kong, J., Bieberich, E., Hernandez, C., Terry, A., Helwa, I., & Liu, Y. (2016). Neutral sphingomyelinase-2 deficiency ameliorates Alzheimer's disease pathology and improves cognition in the 5XFAD mouse. *Journal Of Neuroscience, 36*(33):8653-8667. doi:10.1523/JNEUROSCI.1429-16.2016

73. Dobbing, J. (1974). The Later Growth of the Brain and its Vulnerability. *Pediatrics, 53*(2):2-6

74. Dobrenis, K., Makman, M. H., Stefano, & G. B. (1995). Occurrence of the opiate alkaloid-selective mu3 receptor in mammalian microglia, astrocytes and Kupffer cells. *Brain Research, 686*:239–248.

75. Dong, J., Sulik, K. K., & Chen, S. (2010). The Role of NOX Enzymes in Ethanol-induced Oxidative Stress and Apoptosis in Mouse Embryos. *Toxicology Letters, 193*(1):94. http://doi.org/10.1016/j.toxlet.2009.12.012

76. Dores, R. M. & Baron, A. J. (2011). Evolution of POMC: origin, phylogeny, posttranslational processing, and the melanocortins. *Annals of the New York Academy of Sciences, 1220*:34-48. doi:10.1111/j.1749-6632.2010.05928.x

77. Drago, F., Lombardi, M., Prada, I., Gabrielli, M., Joshi, P., Cojoc, D., Franck, J., Fournier, I., Vizioli, J., & Verderio, C. (2017). ATP Modifies the Proteome of Extracellular Vesicles Released by Microglia and Influences Their Action on Astrocytes. *Frontiers in Pharmacology, 8*(910):1-14.

78. Dunn, K. W., Kamocka, M. M., & McDonald, J. H. (2011). A practical guide to evaluating colocalization in biological microscopy. *American Journal of Physiology - Cell Physiology, 300*(4):C723–C742. http://doi.org/10.1152/ajpcell.00462.2010

79. Emeson, R.B. & Eipper, B.A. (1986). Characterization of pro-ACTH/endorphin-derived peptides in rat hypothalamus. *Journal of Neuroscience, 6*(3):837-849. DOI: 10.1523/JNEUROSCI.06-03-00837.1986

80. Eyo, U., Peng, J., Swiatkowski, P., Mukherjee, A., Bispo, A., & Wu, L. (2014). Neuronal hyperactivity recruits microglial processes via neuronal NMDA receptors and microglial P2Y12 receptors after status epilepticus. *Journal Of Neuroscience, 34(*32), 10528-10540. doi:10.1523/JNEUROSCI.0416-14.2014

81. Fagerberg, L., Hallström, B. M., Oksvold, P., Kampf, C., Djureinovic, D., Odeberg, J., Habuka, M., Tahmasebpoor, S., Danielsson, A., Edlund, K., Asplund, A., Sjöstedt, E., Lundberg, E., Al-Khalili Szigyarto, C., Skogs, M., Takanen, J.O., Berling, H., Tegel, H., Mulder, J., Nilsson, P., Schwenk, J.M., Lindskog, C., Danielsson, F., Mardinoglu, A., Sivertsson, A., von Feilitzen, K., Forsberg, M., Zwahlen, M., Olsson, I., Navani, S., Huss, M., Nielsen, J., Ponten, F., & Uhlén, M. (2014). Analysis of the Human Tissue-specific Expression by Genome-wide Integration of Transcriptomics and Antibody-based Proteomics. *Molecular & Cellular Proteomics : MCP, 13*(2):397–406. http://doi.org/10.1074/mcp.M113.035600

82. Fauré, J., Lachenal, G., Court, M., Hirrlinger, J., Chatellard-Causse, C., Blot, B., Grange, J., Schoehn, G., Goldberg, Y., Boyer, V., Kirchhoff, F., Raposo, G., Garin, J., & Sadoul, R. (2006). Exosomes are released by cultured cortical neurons. *Molecular And Cellular Neuroscience, 31*(4):642-648. doi:10.1016/j.mcn.2005.12.003

83. Fernandez-Lizarbe, S., Pascual, M., & Guerri, C. (2009). Critical role of TLR4 response in the activation of microglia induced by ethanol. *Journal Of Immunology, 183*(7):4733-4744. doi:10.4049/jimmunol.0803590

84. Finer, L.B. & Zolna, M.R. (2014). Shifts in intended and unintended pregnancies in the United States, 2001-2008. *American Journal of Public Health, 104*(Suppl 1):s43-8

85. Fitzner, D., Schnaars, M., van Rossum, D., Krishnamoorthy, G., Dibaj, P., Bakhti, M., Regen, T., Hanisch, U., & Simons, M. (2011). Selective transfer of exosomes from oligodendrocytes to microglia by macropinocytosis. *Journal of Cell Science, 124*(3):447–458

86. Fonseca, M. I., Chu, S.-H., Hernandez, M. X., Fang, M. J., Modarresi, L., Selvan, P., MacGregor, G. R., & Tenner, A. J. (2017). Cell-specific deletion of C1qa identifies microglia as the dominant source of C1q in mouse brain. *Journal of Neuroinflammation, 14*:48. http://doi.org/10.1186/s12974-017-0814-9

87. Francis, J. M., Heyworth, C. M., Spooncer, E., Pierce, A., Dexter, T. M., & Whetton, A. D. (2000). Transforming growth factor-beta 1 induces apoptosis independently of p53 and selectively reduces expression of Bcl-2 in multipotent hematopoietic cells. *Journal of Biological Chemistry, 275*(50):39137-39145.

88. Gallo-Payet, N., Grazzini, E., Coté, M., Chouinard, L., Chorvatova, A., Bilodeau, L., Payet, M.D., & Guillon, G. (1996). Role of Ca2+ in the action of adrenocorticotropin in cultured human adrenal glomerulosa cells. *Journal of Clinical Investigation, 98*:460–6. doi:10.1172/JCI118812

89. Glebov, K., Leochner, M., Jabs, M., Lau, T., Merkel, O., Schloss, P., Steinheauser, C., & Walter, J. (2015). Serotonin Stimulates Secretion of Exosomes from Microglia Cells. *Glia, 63*(4):626–634

90. Grahame-Smith DG, Butcher RW, Ney RL, Sutherland EW. (1967). Adenosine 3',5'-monophosphate as the intracellular mediator of the action of adrenocorticotropic hormone on the adrenal cortex. *Journal of Biological Chemistry, 242*:5535–41.

91. Green PP, McKnight-Eily LR, Tan CH, Mejia R, Denny CH. Vital Signs: Alcohol-Exposed Pregnancies — United States, 2011–2013. MMWR Morb Mortal Wkly Rep 2016;65:91–97. DOI: http://dx.doi.org/10.15585/mmwr.mm6504a6

92. Griffiths, M., Gasque, P., & Neal, J. (2009). The Multiple Roles of the Innate Immune System in the Regulation of Apoptosis and Inflammation in the Brain. *Journal Of Neuropathology And Experimental Neurology, 68*(3):217-226.

93. Gupta, A., & Pulliam, L. (2014). Exosomes as mediators of neuroinflammation. *Journal Of Neuroinflammation, 11*(1):1-20. doi:10.1186/1742-2094-11-68

94. Hadley, M. E. & Haskell-Luevano, C. (1999). The proopiomelanocortin system. *Annual New York Academy of Sciences, 885*(1):1–21.

95. Haorah, J., Ramirez, S. H., Floreani, N., Gorantla, S., Morsey, B., & Persidsky, Y. (2008). Mechanism of alcohol-induced oxidative stress and neuronal injury. *Free*

Radical Biology & Medicine, 45(11), 1542–1550. http://doi.org/10.1016/j.freeradbiomed.2008.08.030

96. He, J. & Crews, F. T. (2008). Increased MCP-1 and Microglia in Various Regions of the Human Alcoholic Brain. *Experimental Neurology, 210*(2):349–358. http://doi.org/10.1016/j.expneurol.2007.11.017

97. Hefendehl, J. K., Neher, J. J., Suhs, R. B., Kohsaka, S., Skodras, A., & Jucker, M. 2014). Homeostatic and injury-induced microglia behavior in the aging brain. *Aging Cell, 13*:60–9.

98. Henderson, G. I., Devi, B. G., Perez, A., & Schenker, S. (1995). In utero ethanol exposure elicits oxidative stress in the rat fetus. *Alcoholism, Clinical and Experimental Research, 19*(3):714–720.

99. Henry, C., Kabbaj, M., Simon, H., LeMoal, M., & Maccari, S. (1994). Prenatal stress increases the hypothalamo-pituitary-adrenal axis response in young and adult rats. *Journal of Neuroendocrinology, 6*(3):341–345.

100. Herman, J.P., Figueiredo, H., Mueller, N.K., Ulrich-Lai, Y., Ostrander, M.M., Choi, D.C., & Cullinan, W.E. (2003). Central mechanisms of stress integration: hierarchical circuitry controlling hypothalamo-pituitary-adrenocortical responsiveness. *Frontiers in Neuroendocrinology, 24*:151–180.

101. Holm, C., Osterlund, T., Laurell, H., & Contreras, J.A. (2000) Molecular mechanisms regulating hormone-sensitive lipase and lipolysis. *Annual Review of Nutrition, 20*:365–93. doi:10.1146/annurev.nutr.20.1.365

102. Hong, S., Beja-Glasser, V., Nfonoyim, B., Frouin, A., Ramakrishnan, S., Merry, K., Stevens, B., Li, S., Shi, Q., Lemere, C., Selkoe, D., Rosenthal, A., & Barres, B. (2016). Complement and microglia mediate early synapse loss in Alzheimer mouse models. *Science, 352*(6286), 712-716. doi:10.1126/science.aad8373

103. Horvath, R. J. & DeLeo, J. A. (2009). Morphine enhances microglial migration through modulation of P2X4 receptor signaling. *Journal of Neuroscience, 29*:998–1005.

104. Jabbar, S., Chastain, L. G., Gangisetty, O., Cabrera, M. A., Sochacki, K., & Sarkar, D. K. (2016). Preconception Alcohol Increases Offspring Vulnerability to Stress. *Neuropsychopharmacology, 41*(11):2782-2793. doi: 10.1038/npp.2016.92

105. Jablonski KA, Gaudet AD, Amici SA, Popovich PG, Guerau-de-Arellano M (2016) Control of the Inflammatory Macrophage Transcriptional Signature by miR-155. *PLOS ONE, 11*(7):1-21. https://doi.org/10.1371/journal.pone.0159724

106. Johnstone R.M., Adam, M., Hammond, J.R., Orr, L., & Turbide, C. (1987). Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). *Journal of Biological Chemistry, 262*(19):9412–9420

107. Joshi, P., Turola, E., Ruiz, A., Bergami, A., Libera, D. D., Benussi, L., Giussani, P., Magnani, G., Comi, G., Legname, G., Ghidoni, R., Furlan, R., Matteoli, M., & Verderio, C. (2014). Microglia convert aggregated amyloid-β into neurotoxic forms through the shedding of microvesicles. *Cell Death & Differentiation, 21*(4):582-593. doi:10.1038/cdd.2013.180

108. Karl, P.I, Gordon, B.H., Lieber, C.S., & Fisher, S.E. (2008) Acetaldehyde production and transfer by the perfused human placental cotyledon. *Science, 242*(4876):273–275.

109. Kavanagh, E. L., Lindsay, S., Halasz, M., Gubbins, L. C., Weiner-Gorzel, K., Guang, M. H. Z., McGoldrick, A., Collins, E., Henry, M., Blanco-Fernández, A., O' Gorman, P., Fitzpatrick, P., Higgins, M. J., Dowling, P., & McCann, A. (2017). Protein and chemotherapy profiling of extracellular vesicles harvested from therapeutic induced senescent triple negative breast cancer cells. *Oncogenesis, 6*(10):e388–. http://doi.org/10.1038/oncsis.2017.82

110. Kelso, M. L., Liput, D. J., Eaves, D. W., & Nixon, K. (2011). Upregulated vimentin suggests new areas of neurodegeneration in a model of an alcohol use disorder. *Neuroscience, 197*:381–393. http://doi.org/10.1016/j.neuroscience.2011.09.019

111. Kettenmann, H., Hanisch, U., Noda, M., & Verkhratsky, A. (2011). Physiology of Microglia. *Physiological Reviews, 91*(2), 461-553.

112. Kim, Y.C., Ariyoshi, N., Artemenko, I., Elliott, M.E., Bhattacharyya, K.K., & Jefcoate, C.R. (1997). Control of cholesterol access to cytochrome P450scc in rat adrenal cells mediated by regulation of the steroidogenic acute regulatory protein. *Steroids, 62*:10–20. doi:10.1016/S0039-128X(96)00153-5

113. Kiss, A. & Aguilera, G. (1992). Participation of alpha 1-adrenergic receptors in the secretion of hypothalamic corticotropin-releasing hormone during stress. *Neuroendocrinology, 56*(2):153-60.

114. Kitamura, T., Feng, Y., Kitamura, Y.I., Chua, S.C. Jr., Xu, A.W., Barsh, G.S., Rossetti, L., & Accili, D. (2006). Forkhead protein FoxO1 mediates Agrp-dependent effects of leptin on food intake. *Nature Medicine, 12*:534-540.

115. Koneru, A., Satyanarayana, S., & Rizwan, S. (2009). Endogenous Opioids: Their Physiological Role and Receptors. *Global Journal of Pharmacology, 3*(3):149-153.

116. Kotkoskie, L. A., and Norton, S. (1989) Cerebral Cortical Morphology and Behavior in Rats Following Acute Prenatal Ethanol Exposure. *Alcohol, Clinincal & Experimental Research, 13*(6):776–781.

117. Kumar, A., Stoica, B. A., Loane, D. J., Yang, M., Abulwerdi, G., Khan, N., Kumar, A., Thom, S. T., & Faden, A. I. (2017). Microglial-derived microparticles mediate neuroinflammation after traumatic brain injury. *Journal of Neuroinflammation, 14*:47. http://doi.org/10.1186/s12974-017-0819-4

118. Lee, S., Imaki, T., Vale, W., & Rivier, C. (1990). Effect of prenatal exposure to ethanol on the activity of the hypothalamic-pituitary-adrenal axis of the offspring: importance of the time of exposure to ethanol and possible modulating mechanisms. *Molecular & Cellular Neuroscience, 1*(2):168–77.

119. Lee, S., Schmidt, D., Tilders, F., & Rivier, C. (2000). Increased activity of the hypothalamicpituitary-adrenal axis of rats exposed to alcohol in utero: role of altered pituitary and hypothalamic function. *Molecular and Cellular Neuroscience, 16*:515–528.

120. Liszewski, M., Kolev, M., Le Friec, G., Leung, M., Bertram, P., Fara, A., Subias, M., Pickering, M., Drouet, C., Meri, S., Arstila, T., Pekkarinen, P., Ma, M., Cope, A., Reinheckel, T., Rodriguez de Cordoba, S., Afzali, B., Atkinson, J., & Kemper, C. (2013). Intracellular Complement Activation Sustains T Cell Homeostasis and Mediates Effector Differentiation. *Immunity, 39*(6), 1143-1157. doi:10.1016/j.immuni.2013.10.018

121. Liu, Y., Kamitakahara, A., Kim, A. J., & Aguilera, G. (2008). Cyclic Adenosine 3′,5′-Monophosphate Responsive Element Binding Protein Phosphorylation Is Required But Not Sufficient for Activation of Corticotropin-Releasing Hormone Transcription. *Endocrinology, 149*(7), 3512–3520. http://doi.org/10.1210/en.2008-0052

122. Llorente A, Skotland T, Sylv¨anne T, Kauhanen D, R´og T, et al. 2013. Molecular lipidomics of exosomes released by PC-3 prostate cancer cells. Biochimica et Biophysica Acta (BBA) - *Molecular and Cell Biology of Lipids, 1831*(7):1302–9

123. Loeffler, J. P., Kley, N., Pittius, C.W., & Hollt, V. (1985). Corticotropin-releasing factor and forskolin increase proopiomelanocortin messenger RNA levels in rat anterior and intermediate cells in vitro. *Neuroscience Letters. 62*:383-387.

124. Logan, R., Wynne, O., Maglakelidze, G., Zhang, C., O'Connell, S., Boyadjieva, N.I., & Sarkar, D.K. (2015). β-endorphin neuronal transplantation into the hypothalamus alters anxiety-like behaviors in prenatal alcohol exposed rats and non-preferring and preferring rats. *Alcoholism: Clinical & Experimental Research, 39*(1):146–157. doi:10.1111/acer.12611.

125. London, A., Cohen, M., & Schwartz, M. (2013). Microglia and monocyte-derived macrophages: Functionally distinct populations that act in concert in CNS plasticity and repair. *Frontiers In Cellular Neuroscience, 7*(34), 1-10 doi:10.3389/fncel.2013.00034

126. Lu, Y., Yeh, W., & Ohashi, P. (2008). LPS/TLR4 signal transduction pathway. *Cytokine, 42*(2):145-151

127. Lupton, C., Burd, L., & Harwood, R. (2004). Cost of fetal alcohol spectrum disorders. *American Journal Of Medical Genetics Part C-Seminars In Medical Genetics, 127C*(1):42-50.

128. Mains, R.E., Eipper, B.A. (1979) Synthesis and secretion of corticotropins, melano-tropins, and endorphins by rat intermediate pituitary cells. *Journal of Biological Chemistry, 254*: 7885–7894.

129. Martin, W. J. & MacIntyre, D. E. (2004). Melanocortin receptors and erectile function. *European Urology, 45*:706–713.

130. Mathew, S. J., Price, R. B., & Charney D. S. (2008). Recent advances in the neurobiology of anxiety disorders: implications for novel therapeutics. *American Journal of Medical Genetics Part C, Seminar in Medical Genetics, 148C*:89-98

131. May, P. A., Chambers, C. D., Kalberg, W. O. (2018). Prevalence of Fetal Alcohol Spectrum Disorders in 4 US Communities. *JAMA, 319*(5):474–482. doi:10.1001/jama.2017.21896

132. May, P. A., Baete, A., Russo, J., Elliott, A. J., Blankenship, J., Kalberg, W. O., Buckley, D., Brooks, M., Hasken, J., Abdul-Rahman, O., Adam, M. P., Robinson, L. K., Manning, M., & Hoyme, H. E. (2014) Prevalence and characteristics of fetal alcohol spectrum disorders. *Pediatrics, 134*(5), 855-866.

133. May, P. A., Gossage, J. P., Kalberg, W. O., Robinson, L. K., Buckley, D., Manning, M., & Hoyme, H. E. (2009) Prevalence and epidemiologic characteristics of FASD from various research methods with an emphasis on recent in-school studies. *Developmental Disabilities Research Reviews, 15*(3):176-192.

134. McClain, J. A., Morris, S. A., Deeny, M. A., Marshall, S. A., Hayes, D. M., Kiser, Z. M., et al. (2011). Adolescent binge alcohol exposure induces long-lasting partial activation of microglia. *Brain Behavior & Immunity, 25*(Suppl. 1):S120–S128

135. McCullough, R. L., McMullen, M. R., Sheehan, M. M., Poulsen, K. L., Roychowdhury, S., Chiang, D. J., Pritchard, M. T., Caballeria, J., & Nagy, L. E. (2018). Complement Factor D protects mice from ethanol-induced inflammation

and liver injury. *American Journal of Physiology Gastrointestinal & Liver Physiology, 315*(1):G66-G79. doi: 10.1152/ajpgi.00334.2017.

136. McNicol, A. M. (1986). A study of intermediate lobe differentiation in the human pituitary gland. *Journal of Pathology, 150*(3):169-73.

137. Michell-Robinson, M. A., Touil, H., Healy, L. M., Owen, D. R., Durafourt, B. A., Bar-Or, A., Antel, J. P., & Moore, C. S. (2015). Roles of microglia in brain development, tissue maintenance and repair. *Brain, 138*(5):1138–1159. http://doi.org/10.1093/brain/awv066

138. Möller, T., Bard, F., Bhattacharya, A., Biber, K., Campbell, B., Dale, E., Eder, C., Gan, L., Garden, G. A., Hughes, Z. A., Pearse, D. D., Staal, R. G., Sayed, F. A., Wes, P. D., & Boddeke, H. W. (2016). Critical data-based re-evaluation of minocycline as a putative specific microglia inhibitor. *Glia, 64*:1788–1794.

139. Momen-Heravi, F., Bala, S., Kodys, K., & Szabo, G. (2015). Exosomes derived from alcohol-treated hepatocytes horizontally transfer liver specific miRNA-122 and sensitize monocytes to LPS. *Scientific Reports, 5*:9991. http://doi.org/10.1038/srep09991

140. Momen-Heravi, F., Kodys, K., & Szabo, G. (2016). MicroRNA Cargo of Extracellular Vesicles from Alcohol-exposed Monocytes Signals Naive Monocytes to Differentiate into M2 Macrophages. *The Journal of Biological Chemistry, 291(*1):149–159. http://doi.org/10.1074/jbc.M115.694133

141. Montecalvo A, Larregina AT, Shufesky WJ, Stolz DB, Sullivan ML, Karlsson, J.M., Baty, C.J., Gibson, G.A., Erdos, G., Wang, Z., Milosevic, J., Tkacheva, O.A., Divito, S.J., Jordan, R., Lyons-Weiler, J., Watkins, S.C., & Morelli, A.E. (2012). Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes. *Blood, 119*(3):756–766

142. Morelli, A.E., Larregina, A.T., Shufesky, W.J., Sullivan, M.L., Stolz, D.B., Papworth, G.D., Zahorchak, A.F., Logar, A.J., Wang, Z., Watkins, S.C., Falo Jr., L.D., & Thomson, A.W. (2004). Endocytosis, intracellular sorting, and processing of exosomes by dendritic cells. *Blood, 104*(10), 3257–3266

143. Mountjoy, K.G. (2015). Pro-Opiomelanocortin (POMC) Neurones, POMC-Derived Peptides, Melanocortin Receptors and Obesity: How Understanding of this System has Changed Over the Last Decade. Journal of *Neuroendocrinology, 27*:406–418

144. Mueller, B., & Bale, T. (2013). Sex-specific programming of offspring emotionality after stress early in pregnancy. *Journal Of Neuroscience, 28*(36), 9055-9065.

145. Mulcahy, L.A., Pink, R.C., & Carter, D.R.F.C. (2014). Routes and mechanisms of extracellular vesicle uptake. *Journal of Extracellular Uptake, 3*(24641):1-14. http://dx.doi.org/10.3402/jev.v3.24641

146. Niswender, K.D., Morton, G.J., Stearns, W.H., Rhodes, C.J., Myers, M.G. Jr., & Schwartz, M.W. Intracellular signaling: Key enzyme in leptin-induced anorexia. *Nature, 413*:794–795.

147. Nixon, K., Kim, D. H., Potts, E. N., He, J., & Crews, F. T. (2008). Distinct cell proliferation events during abstinence after alcohol dependence: microglia proliferation precedes neurogenesis. *Neurobiology of Disease, 31*(2):218–229. http://doi.org/10.1016/j.nbd.2008.04.009

148. Njie, E. G., Boelen, E., Stassen, F. R., Steinbusch, H. W., Borchelt, D. R., & Streit, W. J. (2012). Ex vivo cultures of microglia from young and aged rodent brain reveal age-related changes in microglial function. *Neurobiology of Aging, 33*:195.e1–12.

149. O'Connor, T.M., O'Halloran, D.J., & Shanahan, F. (2000). The stress response and the hypothalamic‐pituitary‐adrenal axis: from molecule to melancholia. *QJM: An International Journal of Medicine, 93*(6):323–333.

150. Ogino, T., Moralejo, D.H., Kose, H., Yamada, T., & Matsumoto, K. (2003). Serum leptin concentration is linked to Chromosomes 2 and 6 in the OLETF rat, an animal model of type 2 diabetes with mild obesity. *Mammalian Genome, 14*(12): 239-244. https://doi.org/10.1007/s00335-003-2295-7

151. Oh, S., Kim, T. K., Hwang, D. S., & Yim, J. (2000). Involvement of retinoblastoma protein p27/kip-1 induced apoptosis. *Cancer Letters, 148*:105–110.

152. Owerbach, D., Rutter, W.J., Roberts, J.L., Whitfeld, P.,Shine, J., Seeburg, P.H., & Shows, T.B. (1981). The proopiocortin (adrenocorticotropin/beta-lipoprotein) gene is located on chromosome 2 in humans. *Somatic Cell Genetics, 7*:359–369.

153. Oyola, M. & Handa, R. (2017). Hypothalamic–pituitary–adrenal and hypothalamic–pituitary–gonadal axes: sex differences in regulation of stress responsivity. *Stress, 20*(5):476-494. doi:10.1080/10253890.2017.1369523

154. Paolicelli, R. C., Bolasco, G., Pagani, F., Maggi, L., Scianni, M., Panzanelli, P., Giustetto, M., Ferreira, T. A., Guiducci, E., Dumas, L., Ragozzino, D, & Gross, C. T. (2011). Synaptic pruning by microglia is necessary for normal brain development. *Science, 333*(6048), 1456-1458. doi: 10.1126/science.1202529

155. Parton, L.E., Ye, C.P., Coppari, R., Enriori, P.J., Choi, B., Zhang, C.Y., Xu, C., Vianna, C.R., Balthasar, N., Lee, C.E., Elmquist, J.K., Cowley, M.A., &

Lowell, B.B. (2007). Glucose sensing by POMC neurons regulates glucose homeostasis and is impaired in obesity. *Nature, 449*:228–232

156. Pascual, M., Montesinos, J., Montagud-Romero, S., Forteza, J., Rodríguez-Arias, M., Miñarro, J., & Guerri, C. (2017). TLR4 response mediates ethanol-induced neurodevelopment alterations in a model of fetal alcohol spectrum disorders. *Journal of Neuroinflammation, 14*:145. http://doi.org/10.1186/s12974-017-0918-2

157. Perry, V. H., Matyszak, M. K., & Fearn, S. (1993). Altered antigen expression of microglia in the aged rodent CNS. *Glia, 7*:60–7.

158. Petersen, S.V., Thiel, S., Jensen, L., Vorup-Jensen, T., Koch, C., & Jensenius, J.C. (2000) Control of the classical and the MBL pathway of complement activation. *Molecular Immunology, 37*(14), 803–811.

159. Pintar JE, Schachter BS, Herman AB, Durgerian S & Krieger DT 1984 Characterization and localization of proopiomelanocortin messenger RNA in the adult rat testis. *Science, 225*:632–634.

160. Porcu, P., Grana, X., Li, S., Swantek, J., De Luca, A., Giordano, A., & Baserga, R. (1994). An E2F binding sequence negatively regulates the response of the insulin-like growth factor I (IGF-I) promoter to simain virus 40T antigen and to serum. *Oncogene, 9*:2125–2134.

161. Porter, A. G. & Jänicke, R. U. (1999). Emerging roles of caspase-3 in apoptosis. *Cell Death & Differentiation, 6*(2):99-104.

162. Potolicchio, I., Carven, G.J., Xu, X., Stipp, C., Riese, R.J., Stern, L.J., & Santambrogio, L. (2005). Proteomic analysis of microglia-derived exosomes: metabolic role of the aminopeptidase CD13 in neuropeptide catabolism. *Journal of Immunology, 175*(4):2237-43.

163. Pritchard, L.E., A.V. Turnbull, & A. White, (2002). Pro-opiomelanocortin processing in the hypothalamus: impact on melanocortin signalling and obesity. *Journal of Endocrinology, 172*(3):411-21.

164. Qin, L., & Crews, F. T. (2012). Chronic ethanol increases systemic TLR3 agonist-induced neuroinflammation and neurodegeneration. *Journal of Neuroinflammation, 9*:130. http://doi.org/10.1186/1742-2094-9-130

165. Qin, L., & Crews, F.T. (2012), NADPH oxidase and reactive oxygen species contribute to alcohol-induced microglial activation and neurodegeneration *Journal of Neuroinflammation, 9*(5):1-19. doi: 10.1186/1742-2094-9-5

166. Raffin-Sanson, M.L., de Keyzer, Y., & Bertagna, X (2003). Proopiomelanocortin, a polypeptide precursor with multiple functions: from

physiology to pathological conditions. *European Journal of Endocrinology, 149:*79–90

167. Rahpeymai, Y., Hietala, M., Wilhelmsson, U., Fotheringham, A., Davies, I., Nilsson, A., Zwirner, J., Wetsel, R.A., Gerard, C., Pekny, M., & Pekna, M. (2006). Complement: a novel factor in basal and ischemia-induced neurogenesis. *EMBO Journal, 25*(6), 1364-1374.

168. Rajendran, L., Honsho, M., Zahn, T.R., Keller, P., Geiger, K.D., Verkade, P., Simons, K. (2006). Alzheimer's disease beta-amyloid peptides are released in association with exosomes. *Proceeding from the National Academies of Science, 103*(30), 11172–11177.

169. Ransohoff, R. M. & Perry, V. (2009). Microglial Physiology: Unique Stimuli, Specialized Responses. *Annual Review Of Immunology, 27*:119-145.

170. Ransohoff, R. M. (2011). Microglia and monocytes: 'tis plain the twain meet in the brain. *Nature Neuroscience, 14*(9), 1098-1100. doi:10.1038/nn.2917

171. Risher, W. C., Ustunkaya, T., Singh Alvarado, J., & Eroglu, C. (2014). Rapid Golgi analysis method for efficient and unbiased classification of dendritic spines. *PLoS One, 9*(9), e107591. doi: 10.1371/journal.pone.0107591

172. Robbins, L.S., Nadeau, J.H., Johnson, K.R., Kelly, M.A., Roselli-Rehfuss, L., Baack, E., Mountjoy, K.G., & Cone, R.D. (1993). Pigmentation phenotypes of variant extension locus alleles result from point mutations that alter MSH receptor function. *Cell, 72*:827–834.

173. Roberts, J. L., Budarf, M.J., Baxter, J.D., & Herbert, E. (1979). Selective reduction of proadrenocorticotropin/endorphin protein and messenger ribonucleic acid activity in mouse pituitary tumor cells by glucocorticoids. *Biochemistry 18*:4907-491 5.

174. Roberts, J.L., Lundbald, J.R., Eberwine, J.H., Fremeau, R.T., Salton, S.R., & Blum, M. (1987). Hormonal regulation of POMC gene expression in pituitary. *Annals New York Academy Of Sciences, 512*:275-85.

175. Roychowdhury, S., McMullen, M., Pritchard, M., Hise, A., van Rooijen, N., Medof, M., Stavitsky, A. B., & Nagy, L. (2010). An Early Complement-Dependent and TLR-4-Independent Phase in the Pathogenesis of Ethanol-Induced Liver Injury in Mice. *Hepatology, 49*(4), 1326-1334.

176. Rus, H., Cudrici, C., & Niculescu, F. (2005) The role of the complement system in innate immunity. *Immunologic Research, 33*(2), 103-112.

177. Saavedra, H. I., Wu, L., de Bruin, A., Timmers, C., Rosol, T. J., Weinstein, M., Robin-son, M. L., & Leone, G. (2002). Specificity of E2F1, E2F2,

and E2F3 in mediatingphenotypes induced by loss of Rb. *Cell Growth & Differentiation, 13*:215–225.

178. Saito, M., Chakraborty, G., Hui, M., Masiello, K., & Saito, M. (2016). Ethanol-Induced Neurodegeneration and Glial Activation in the Developing Brain. *Brain Sciences, 6*(3):31. http://doi.org/10.3390/brainsci6030031

179. Saito, M., Chakraborty, G., Shah, R., Mao, R. F., Kumar, A., Yang, D. S., et al. (2012). Elevation of GM2 ganglioside during ethanol-induced apoptotic neurodegeneration in the developing mouse brain. *Journal of Neurochemistry 121*:649–661.

180. Sampson, P. D., Streissguth, A. P., Bookstein, F. L., Little, R. E., Clarren, S. K., Dehaene, P., Hanson, J. W., & Graham, J. M. Jr. (1997). Incidence of fetal alcohol syndrome and prevalence of alcohol-related neurodevelopmental disorder. *Teratology, 56*:317–326

181. Sánchez-Capelo A. (2005). Dual role for TGF-beta1 in apoptosis. *Cytokine Growth Factor, 16*(1):15-34.

182. Sarkar, D. K., Kuhn, P., Marano, J., Chen, C., & Boyadjieva, N. (2007). Alcohol exposure during the developmental period induces beta-endorphin neuronal death and causes alteration in the opioid control of stress axis function. *Endocrinology, 148*(6), 2828-2834.

183. Sarkar, D. K., Sengupta, A., Zhang, C., Boyadjieva, N., & Murugan, S. (2012). Opiate Antagonist Prevents μ- and δ-Opiate Receptor Dimerization to Facilitate Ability of Agonist to Control Ethanol-altered Natural Killer Cell Functions and Mammary Tumor Growth. *Journal of Biological Chemistry, 287*(20), 16734–16747. http://doi.org/10.1074/jbc.M112.347583

184. Sarkar, D. K. (2015). Neuroendocrinology. Unpublished Manuscript, Rutgers University, The State University of New Jersey, New Brunswick, NJ.

185. Schafer, D. P., Lehrman, E. K., Kautzman, A. G., Koyama, R., Mardinly, A. R., Yamasaki, R., Ransohoff, R. M., Greenberg, M. E., Barres, B. A., & Stevens, B. (2012). Microglia Sculpt Postnatal Neural Circuits in an Activity and Complement-Dependent Manner. *Neuron*, *74*(4), 691–705. http://doi.org/10.1016/j.neuron.2012.03.026

186. Sebastian, B., Roychowdhury, S., Tang, H., Hillian, A., Feldstein, A., Stahl, G., Takahashi, K., & Nagy, L. (2011). Identification of a Cytochrome P4502E1/Bid/C1q-dependent Axis Mediating Inflammation in Adipose Tissue after Chronic Ethanol Feeding to Mice. *Journal Of Biological Chemistry, 286*(41), 35989-35997.

187. Sharples, R. A., Vella, L. J., Nisbet, R. M., Naylor, R., Perez, K., Barnham, K. J., Masters, C. L., & Hill, A. F. (2008). Inhibition of γ--secretase

causes increased secretion of amyloid precursor protein C-terminal fragments in association with exosomes. *FASEB Journal, 22*(5):1469–1478

188. Shrivastava, P., Cabrera, M. A., Chastain, L. G., Boyadjieva, N. I., Jabbar, S., Franklin, T., & Sarkar, D. K. (2017). Mu-opioid receptor and delta-opioid receptor differentially regulate microglial inflammatory response to control proopiomelanocortin neuronal apoptosis in the hypothalamus: effects of neonatal alcohol. *Journal Of Neuroinflammation, 14*(1):83-102. doi:10.1186/s12974-017-0844-3

189. Shyng, S.L. & Nichols, C.G.. (1998). Membrane phospholipid control of nucleotide sensitivity of KATP channels. *Science, 282*:1138–1141

190. Silverman, S. M., Kim, B.-J., Howell, G. R., Miller, J., John, S. W. M., Wordinger, R. J., & Clark, A. F. (2016). C1q propagates microglial activation and neurodegeneration in the visual axis following retinal ischemia/reperfusion injury. *Molecular Neurodegeneration, 11*:24. http://doi.org/10.1186/s13024-016-0089-0

191. Spät, A., Hunyady, L., & Szanda, G. (2016). Signaling Interactions in the Adrenal Cortex. *Frontiers in Endocrinology, 7*(17):1-10. http://doi.org/10.3389/fendo.2016.00017

192. Steele, M.R., Inman, D.M., Calkins, D.J., Horner, P.J., & Vetter, M.L. (2006). Microarray analysis of retinal gene expression in the DBA/2J model of glaucoma. *Investigative Ophthalmology & Visual Science, 47*(1), 977–985

193. Stephan, A., Barres, B., & Stevens, B. (2012). The complement system: An unexpected role in synaptic pruning during development and disease. *Annual Review of Neuroscience, 35*:369–389.

194. Stephan, A., Madison, D., Mateos, J., Fraser, D., Lovelett, E., Coutellier, L., Kim, L., Tsai, H., Huang, E. J., Rowitch, D. H., Berns, D. S., Tenner, A. J., Shamloo, M., & Barres, B. (2013). A Dramatic Increase of C1q Protein in the CNS during Normal Aging. *Journal Of Neuroscience, 33*(33), 13460-13474.

195. Stephens, M.A. (2012). Physiological Functions of Glucocorticoids in Stress and Their Relation to Pharmacological Actions. *Alcohol Research, 34*(4): 468–483

196. Stevens, B., Allen, N. J., Vazquez, L. E., Howell, G. R., Christopherson, K. S., Nouri, N., Micheva, K. D., Mehalow, A. K., Huberman, A. D., Stafford, B., Sher, A., Litke, A. M., Lambris, J. D., Smith, S. J. John, S. W. M., Barres, B. A. (2007). The Classical Complement Cascade Mediates CNS Synapse Elimination. *Cell, 131*(6), 1164-1178. doi: https://doi.org/10.1016/j.cell.2007.10.036

197. Streit, W. J., Mrak, R. E., & Griffin, W. S. T. (2004). Microglia and neuroinflammation: a pathological perspective. *Journal of Neuroinflammation, 1*:14. http://doi.org/10.1186/1742-2094-1-14

198. Stuendl, A., Kunadt, M., Kruse, N., Bartels, C., Moebius, W., Danzer, K. M., … Schneider, A. (2016). Induction of α-synuclein aggregate formation by CSF exosomes from patients with Parkinson's disease and dementia with Lewy bodies. *Brain, 139*(2), 481–494. http://doi.org/10.1093/brain/awv346

199. Substance Abuse and Mental Health Services Administration. (2014). Results from the 2013 National Survey on Drug Use and Health: Summary of National Findings, NSDUH Series H-48, HHS Publication No. (SMA) 14-4863. Retrieved from https://www.samhsa.gov/data/sites/default/files/NSDUHresultsPDFWHTML2013/Web/NSDUHresults2013.pdf

200. Svensson, K.J., Christianson, H.C., Wittrup, A., Bourseau-Guilmain, E., Lindqvist, E., Svensson, L.M., Mörgelin, M., & Belting, M. (2013). Exosome uptake depends on ERK1/2-heat shock protein 27 signaling and lipid Raft-mediated endocytosis negatively regulated by caveolin-1. *Journal of Biological Chemistry, 288*(24),17713–17724

201. Takahashi, L. K., & Kalin, N. H. (1991). Early developmental and temporal characteristics of stress-induced secretion of pituitary-adrenal hormones in prenatally stressed rat pups. *Brain Research, 558*(1), 75-78. http://dx.doi.org/10.1016/0006-8993(91)90715-8

202. Tam, J., Bidgood, S., McEwan, W., & James, L. (2014). Intracellular sensing of complement C3 activates cell autonomous immunity. *Science, 345*(6201), doi:10.1126/science.1256070

203. Tan, C. H., Denny, C. H., Cheal, N. E., Sniezek, J. E., & Kanny, D. (2015). Alcohol use and binge drinking among women of childbearing age - United States, 2011-2013. MMWR: Morbidity & Mortality Weekly Report, 64(37), 1042-1046. doi:10.15585/mmwr.mm6437a3

204. Tang, Y. & Le, W. (2016). Differential roles of M1 and M2 microglia in neurodegenerative diseases. *Molecular Neurobiology. 53*:1181–1194. 10.1007/s12035-014-9070-5

205. Ten, V., Yao, J., Ratner, V., Stark, R., Polin, R., Sosunov, S., Silverstein, S., Fraser, D., Botto, M., Sivasankar, B., Morgan, B., Pinsky, D., Vannucci, S., & Starkov, A. (2010). Complement component C1q mediates mitochondria-driven oxidative stress in neonatal hypoxic-ischemic brain injury. *Journal Of Neuroscience, 30*(6), 2077-2087. doi:10.1523/JNEUROSCI.5249-09.2010

206. Thurman, J., & Holers, V. (2006). The central role of the alternative complement pathway in human disease. *Journal Of Immunology, 176*(3), 1305-1310.

207. Tian, X., Guo, J., Zhu, M., Li, M., Wu, G., & Xia, Y. (2013). δ-Opioid Receptor Activation Rescues the Functional TrkB Receptor and Protects the Brain from Ischemia-Reperfusion Injury in the Rat. *PLoS ONE, 8*(7), e69252. http://doi.org/10.1371/journal.pone.0069252

208. Toda, C., Santoro, A., Kim, J. D., & Diano, S. (2017). POMC Neurons: From Birth to Death. *Annual Review of Physiology, 79*:209–236. http://doi.org/10.1146/annurev-physiol-022516-034110

209. Tremblay, M., Stevens, B., Sierra, A., Wake, H., Bessis, A., & Nimmerjahn, A. (2011). The role of microglia in the healthy brain. *The Journal Of Neuroscience, 31*(45):16064-16069. doi:10.1523/JNEUROSCI.4158-11.2011

210. Tsigosa,,C. & Chrousos, G.P. (2002). Hypothalamic–pituitary–adrenal axis, neuroendocrine factors and stress. *Journal of Psychosomatic Research, 53*:865-871.

211. Turchan-Cholewo, J., Dimayuga, F. O., Ding, Q., Keller, J. N., Hauser, K. F., Knapp, P. E., & Bruce-Keller, A. J. (2008). Cell-specific actions of HIV-Tat and morphine on opioid receptor expression in glia. *Journal of Neuroscience Research, 86*(9):2100–2110. http://doi.org/10.1002/jnr.21653

212. US Department of Health and Human Services. US Surgeon General releases advisory on alcohol use in pregnancy. Washington, DC: US Department of Health and Human Services; 2005. https://wayback.archive-it.org/3926/20140421162517/http://www.surgeongeneral.gov/news/2005/02/sg02222005.html

213. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, & National Institute on Alcohol Abuse and Alcoholism. (2000). Tenth Special Report to Congress on Alcohol and Health. Retrieved from: https://pubs.niaaa.nih.gov/publications/10report/10thspecialreport.pdf

214. U.S. Department of Health and Human Services and U.S. Department of Agriculture. 2015 – 2020 Dietary Guidelines for Americans. 8th Edition. December 2015. Available at https://health.gov/dietaryguidelines/2015/guidelines/.

215. Vaisse, C., Halaas, J.L., Horvath, C.M., Darnell, J.E. Jr., Stoffel, M., & Friedman, J.M. (1996). Leptin activation of Stat3 in the hypothalamus of wild-type and ob/ob mice but not db/db mice. *Nature Genetics, 14*:95–97.

216. Valadi, H., Ekström, K., Bossios, A., Sjöstrand, M., Lee, J. J. & Lötvall, J. O. (2007). Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nature Cell Biology, 9*:654–659.

217. Veerhuis, R., Janssen, I., De Groot, C.J., Van Muiswinkel, F.L., Hack, C.E., & Eikelenboom, P. (1999). Cytokines associated with amyloid plaques in Alzheimer's disease brain stimulate human glial and neuronal cell cultures to secrete early complement proteins, but not C1-inhibitor. *Experimental Neurology, 160*(1), 289–299.

218. Vetreno, R. P., Qin, L., & Crews, F. T. (2013). Increased receptor for advanced glycation end product expression in the human alcoholic prefrontal cortex is linked to adolescent drinking. *Neurobiology Of Disease, 59*:52-62. doi:10.1016/j.nbd.2013.07.002

219. Wake, H., Moorhouse, A. J., Jinno, S., Kohsaka, S., & Nabekura, J. (2009). Resting microglia directly monitor the functional state of synapses in vivo and determine the fate of ischemic terminals. *Journal of Neuroscience, 29*(13), 3974-3980. doi: 10.1523/JNEUROSCI.4363-08.2009

220. Walker, D. G., & Lue, L.-F. (2015). Immune phenotypes of microglia in human neurodegenerative disease: challenges to detecting microglial polarization in human brains. *Alzheimer's Research & Therapy, 7*(1):56. http://doi.org/10.1186/s13195-015-0139-9

221. Waltman R. & Iniquez, E.S. (1972). Placental transfer of ethanol and its elimination at term. *Obstetrics & Gynecology, 40*(2):180–185.

222. Wang X., Zhanga, K., Yanga, F., Rena, Z., Xua, M., Franka, J. A., Kec, Z., & Luo, J. (2018). Minocycline protects developing brain against ethanol-induced damage. *Neuropharmacology, 129*:84–99.

223. Wang, C.C., Wu, C.H., Shieh, J.Y., and Wen, C.Y. (2002). Microglial distribution and apoptosis in fetal rat brain. *Brain Research & Developing Brain Research. 139*, 337–342.

224. Wang, G., Dinkins, M., He, Q., Zhu, G., Poirier, C., Campbell, A., Mayer-Proschel, M., & Bieberich, E. (2012). Astrocytes secrete exosomes enriched with proapoptotic ceramide and prostate apoptosis response 4 (PAR-4): potential mechanism of apoptosis induction in Alzheimer disease (AD). *Journal of Biological Chemistry, 287*(25), 21384–21395.

225. Weinberg, J., Sliwowska, J. H., Lan, N., & Hellemans, K. C. (2008). Prenatal Alcohol Exposure: Foetal Programming, the Hypothalamic-Pituitary-Adrenal Axis and Sex Differences in Outcome. *Journal Of Neuroendocrinology, 20*(4), 470-488. doi:10.1111/j.1365-2826.2008.01669.x

226. West, J. R., Hamre, K. M., Cassell, B. A. & Cassell, M. D. (1986). Effects of Ethanol Exposure during the Third Trimester Equivalent on Neuron Number in Rat Hippocampus and Dentate Gyrus. *Alcoholism: Clinical and Experimental Research, 10*(2):190-197.

227. Williams, K., Bar-Or, A., Ulvestad, E., Olivier, A., Antel, J. P., Yong, V. W. (1992). Biology of adult human microglia in culture: comparisons with peripheral blood monocytes and astrocytes. *Journal of Neuropathology & Experimental Neurology, 51*:538–49.

228. Wilms, H., Sievers, J., Rickert, U., Rostami-Yazdi, M., Mrowietz, U., & Lucius, R. (2010). Dimethylfumarate inhibits microglial and astrocytic inflammation by suppressing the synthesis of nitric oxide, IL-1beta, TNF-alpha and IL-6 in an in-vitro model of brain inflammation. *Journal Of Neuroinflammation, 7*(30). doi:10.1186/1742-2094-7-30

229. Wu, D., Jackson-Lewis, V., Vila, M., Tieu, K., Teismann, P., Vadseth, C., Choi, D.K, Ischiropoulos, H., & Przedborski, S. (2002). Blockade of microglial activation is neuroprotective in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson disease. *Journal Of Neuroscience, 22*(5):1763–1771.

230. Wu, Y., Deng, W., & Klinke, D. J. (2015). Exosomes: Improved methods to characterize their morphology, RNA content, and surface protein biomarkers. *The Analyst, 140*(19):6631–6642. http://doi.org/10.1039/c5an00688k

231. Wu, Y., Lousberg, E.L., Moldenhauer, L.M., Hayball, J.D., Robertson, S.A., Coller, J.K., Watkins, L.R., Somogyi, A.A., & Hutchinson, M.R. (2011). Attenuation of microglial and IL-1 signaling protects mice from acute alcohol-induced sedation and/or motor impairment. *Brain Behavior Immunity, 25*(S1): S155-S164.

232. Yan, Q., Zhang, J., Liu, H., Babu-Khan, S., Vassar, R., Biere, A., Citron, M., & Landreth, G. (2003). Anti-inflammatory drug therapy alters β-amyloid processing and deposition in an animal model of Alzheimer's disease. *Journal Of Neuroscience, 23*(20), 7504-7509.

233. Yang, J. Y., Xue, X., Tian, H., Wang, X. X., Dong, Y. X., Wang, F., Zhao, Y. N., Yao, X. C., Cui, W., & Wu, C. F. (2014). Role of microglia in ethanol-induced neurodegenerative disease: pathological and behavioral dysfunction at different developmental stages. *Pharmacology & Therapeutics, 144*:321–337. 10.1016/j.pharmthera.2014.07.002

234. Yang, Y., Xia, X., Zhang, Y., Wang, Q., Li, L., Luo, G., & Xia, Y. (2009). δ-Opioid receptor activation attenuates oxidative injury in the ischemic rat brain. *BMC Biology, 7*:55. http://doi.org/10.1186/1741-7007-7-55

235. Yeo, G.S.H., Farooqi, I.S., Challis, B.G., Jackson, R.S., & O'Rahilly, S. (2000). The role of melanocortin signalling in the control of body weight:

evidence from human and murine genetic models. *Quarterly Journal of Medicine, 93*:7–14.

236. Yu, J. T., Foster, R. G., Dean, D. C. (2001). Transciptional repression by RB-E2Fand *regulation of anchorage-independent survival.* Molecular Cell Biology, 21:3325–3335

237. Zhang, K., Wang, H., Xu, M., Frank, J. A., & Luo, J. (2018). Role of MCP-1 and CCR2 in ethanol-induced neuroinflammation and neurodegeneration in the developing brain. *Journal of Neuroinflammation, 15*:197. http://doi.org/10.1186/s12974-018-1241-2

238. Zhang, X., Sliwowska, J. H., & Weinberg, J. (2005). Prenatal alcohol exposure and fetal programming: Effects on neuroendocrine and immune function. *Experimental Biology and Medicine, 230*(6):376-388.

239. Zhao, Y. N., Wang, F., Fan, Y. X., Ping, G. F., Yang, J. Y., & Wu, C. F. (2013). Activated microglia are implicated in cognitive deficits, neuronal death, and successful recovery following intermittent ethanol exposure. *Behavior and Brain Research 236*:270–282.

240. Zhou, A., Bloomquist, B.T., & Mains, R.E. (1993). The prohormone convertases PC1 and PC2 mediate distinct endoproteolytic cleavages in a strict temporal order during proopiomelanocortin biosynthetic processing. *Journal of Biological Chemistry, 268*(3):1763-9.