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THE IMMEDIATE AND LONG TERM EFFECTS OF MICROGLIAL CELL ACTIVATION ON STRESS RESPONSE IN FETAL ALCOHOL EXPOSURE MODEL

By

TINA CARLA FRANKLIN

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Dipak Sarkar, D. Phil., Ph.D.

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ABSTRACT OF THE DISSERTATION

The Immediate and Long Term Effects of Microglial Cell Activation on Stress Response in Fetal Alcohol Exposure Model

By TINA CARLA FRANKLIN

Dissertation Director:

Dipak Sarkar, D. Phil., Ph.D.

Fetal alcohol exposure has many detrimental effects on the developing fetus that can result in fetal alcohol spectrum disorder (FASD). The developing CNS is particularly sensitive to ethanol neurotoxic effects and exposure during the fetal period results in increased neuronal damage and cell death. Furthermore, many of FASD patients display lifelong stress response abnormalities involving alterations in hypothalamic-pituitary-adrenal (HPA) response. Microglia, which are long lived self-replenishing CNS immune cells, have been shown to contribute to ethanol induced neurotoxicity through the release of pro-inflammatory cytokines and reactive oxygen species. We found that ethanol exposure during the early postnatal period, which is equivalent to the third trimester in humans, significantly increases microglial activation and expression of pro-inflammatory cytokines and chemokines in the mediobasal hypothalamus of neonates. Minocycline, an inhibitor of microglial activation, prevented ethanol induced microglial activation and production of pro-inflammatory cytokines in neonates. Moreover, microglial activation during the neonatal period permanently reduced the number of hypothalamic β endorphin (BEP) expressing neurons, an endogenous opioid involved in immune and stress regulation. We provide supporting evidence that the microglia mediated reduction of BEP neurons may contribute to HPA hyper response to acute restraint stress in ethanol exposed animals.

Microglia can initiate and perpetuate stress activation by releasing pro-inflammatory cytokine. It has been shown that microglial stimulation and activation can alter their responses to subsequent stimuli. We found that neonatal ethanol exposure activates hypothalamic microglia and programs them to become more sensitize to subsequent stimuli in adulthood. We demonstrate that neonatal ethanol exposed adult males are hyper-responsive to acute restraint stress and LPS exposure as quantified by the increase in plasma CORT and ACTH levels. This change correlated with an increase expression of the pro-inflammatory cytokine TNF- α in the PVN. Minocycline pre-treatment during neonatal ethanol exposure prevented the increased expression of TNF- α and the hyper response to immune challenge in alcohol fed adult males. This study demonstrates that ethanol exposure during development results in long term alterations in stress response partially due to over sensitized microglia.

DEDICATION

I dedicate this work to my wonderful family, especially my mother Sheila and my partner Jesse, for their unconditional love and support. Their love and encouragement have made this journey possible.

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LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
AD	Ad libitum
AF	Alcohol fed
ARBD	Alcohol related birth defects
ARC	Arcuate Nucleus
ARND	Alcohol related neurobehavioral disorders
BBB	Blood Brain Barrier
BEP	B-endorphin
CAT	Catalase
CC	Cortical Cells
CD	Cluster of differentiation molecule
CDC	Center for Disease Control and Prevention
CNS	Central nervous system
CORT	Corticosterone
CRH	Corticotropin-releasing hormone
DAMP	Damage associated molecular pattern
DOR	Delta opioid receptor
E. coli	Escherichia coli
FAS	Fetal alcohol syndrome
FASD	Fetal alcohol spectrum disorder
GPX	Glutathione peroxidase
GR	Glucocorticoid receptor Type II
HMGB1	High motility group box 1
HPA	Hypothalamic-pituitary-adrenal axis
IL	Interleukin

LPS	Lipopolysaccharide
М	Minocycline
MBH	Mediobasal hypothalamus
ME	Median eminence
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MOR	Mu opioid receptor
MR	Mineralocorticoid receptor Type I
PAMP	Pathogen associated molecular pattern
PD	Postnatal day
PF	Pair fed
Poly I:C	Polyinosinic:polycytidylic acid
POMC	Proopiomelanocortin
PRR	Pattern recognition receptor
PVN	Paraventricular nucleus
ROS	Reactive oxygen species
SEM	Standard Error of Measurement
SHRP	Stress hyporesponsive period
SOD	Superoxide dismutase
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha

CHAPTER 1 BACKGROUND OVERVIEW

1.1 FETAL ALCOHOL SPECTRUM DISORDER

The Center for Disease Control and Prevention (CDC) report that more than half (52%) of women of childbearing age, 18-44 years old, regularly consume alcohol. Of these women, 15% report regularly engaging in binge drinking which is defined as four or more drinks on an occasion for women. It has been reported that as many as 50% of all pregnancies in the United States are unplanned therefore the risk of ethanol consumption during the early stages of pregnancy are high. Despite various efforts to increase awareness to the potential risks of ethanol exposure during development, it has been reported that as a minimum 7.6% of pregnant women continue consuming alcohol during pregnancy and at least 1.4% of pregnant alcohol drinking women engage in regular episodes of binge drinking throughout their pregnancy. (Fetal alcohol spectrum disorders, Data and Statistics, CDC, 2012)

Ethanol consumption during pregnancy results in teratogenic effects on the developing fetus. The range of adverse effects that can result from prenatal ethanol exposure is collectively termed Fetal Alcohol Spectrum Disorder (FASD). The most severely affected FASD patients are born with Fetal Alcohol Syndrome (FAS). FAS patients display a number of physical and developmental anomalities including facial dysmorphology, pre- and postnatal growth deficiencies and central nervous system (CNS) dysfunction (Alfonso-Loeches and Guerri, 2011; Fukui and Sakata-Haga, 2009). The diagnosis for FAS requires all three characteristics (Goodlett and Johnson, 1997; Fukui and Sakata-Haga, 2009). Prenatal ethanol exposure can also results in numerous alcohol-related effects that do not produce full FAS, including alcohol related birth defects (ARBD), alcohol related neurobehavioral disorders (ARND) and other alcohol-related effects (Hellemans et al., 2010). It is estimated that the prevalence of FAS is as high as 2.0 cases per 1000 live births in the US. Moreover, the prevalence of FASD is estimated to be at least three times as many (Medina and Krahe, 2008).

The outcomes of prenatal ethanol exposure vary on a series of biological and environmental factors. The most important factors appear to be the dose, the pattern and the developmental timing of ethanol exposure (Goodlett and Johnson, 1997; Alfonso-Loeches and Guerri, 2011). It is well documented that higher doses of ethanol exposure result in more severe effects on the developing fetus. Binge drinking, which is defined as large amounts of ethanol consumption during a short period of time, produces high blood alcohol levels (BAL \geq 200 mg/dL) (Alfonso-Loeches and Guerri, 2011). Studies show that episodes of binge drinking result in the most severe effects of prenatal ethanol exposure (Goodlett and Johnson, 1997; Alfonso-Loeches and Guerri, 2011). This effect is consistently observed throughout clinical and animal studies (Alfonso-Loeches and Guerri, 2011).

The pattern of ethanol exposure also greatly affects the severity of alcohol related effects. It has been shown that longer periods of ethanol exposure at lower concentrations result in less severe effects than equivalent amounts at high concentrations for shorter periods (Goodlett and Johnson, 1997). However, longer periods of exposure to ethanol increase the range of ethanol induced defects in the developing fetus (O'leary et al., 2010).

Lastly, the developmental timing of ethanol exposure results in differential effects due to specific developmental periods (Weinberg et al., 2008; Medina, 2011; Alfonso-Loeches and Guerri, 2011). The first trimester is the major period of organogenesis. Ethanol exposure during the first trimester can result in neural tube and crest defects and FAS associated facial dismorphology (Medina, 2011; Alfonso-Loeches and Guerri, 2011). The second trimester corresponds to the embryonic period when organogenesis and differentiation are completed (Weinberg et al., 2008). This period is also associated with marked growth spurt (Weinberg et al., 2008). Ethanol exposure during this period results in abnormal cell differentiation and migration as well as growth retardation (Weinberg et al., 2008; Alfonso-Loeches and Guerri, 2011). Lastly, the third trimester corresponds to the late embryonic stage and fetal period when a drastic brain growth spurt occurs (Weinberg et al. 2008; Medina, 2011; Alfonso-Loeches and Guerri, 2011).

Ethanol exposure during the third trimester has been associated with increased apoptotic and necrotic cell death as well as defects in synaptogenesis and neuronal plasticity (Sarkar et al., 2008; Medina, 2011; Alfonso-Loeches and Guerri, 2011). The developmental defects of prenatal alcohol exposure described above are compiled in Figure 1(Alfonso-Loeches and Guerri, 2011).



FIGURE 1. DEVELOPMENTAL EFFECTS OF ALCOHOL DURING CENTRAL NERVOUS SYSTEM ONTOGENY IN HUMANS AND RATS

Abbreviations: NSCs, neural stem cells; F, fetal; P, postnatal. Adapted from Alfonso-Loeches and Guerri 2011

Although the outcomes of prenatal ethanol exposure vary throughout the spectrum, FASD patients consistently display cognitive, neuropsychological and behavioral deficits (Hellemans et al., 2010; Alfonso-Loeches and Guerri, 2011). Human and animal studies demonstrate that prenatal ethanol exposure is often associated with deficits in learning and memory, anxiety, depression, hyperactivity, hyperesponsitivity to stressors, and deficits in response inhibition (Hellemans et al., 2010; Alfonso-Loeches and Guerri, 2011, Kane et al., 2012). These effects are thought to be due to hypothalamic pituitary adrenal (HPA) abnormalities in response or inhibition. Ethanol exposure during the second and/or third trimester has been shown to increase abnormal HPA response (Sarkar et al., 2007; Weinberg et al., 2008; Hellemans et al., 2010). Moreover, prenatal ethanol exposure is thought to increase vulnerability to permanent alterations in innate immune function due to fetal programming which may contribute to HPA dysfunction (Arjona et al., 2006; Boyadjieva et al., 2009; Sarkar et al. 2012, Bodnar and Weinberg, 2013).

1.2 HYPOTHALAMIC PITUITARY ADRENAL AXIS

The hypothalamus, pituitary and adrenal cortex make up the primary components of the HPA axis. The HPA axis is vital for proper development and biobehavioral responses to stressors. When an individual is presented with a change in homeostasis that may yield a potential danger (whether it is real or perceived), the HPA axis is activated to properly respond to the stimulus. Initiation of HPA activation is the same regardless if the stimulus is caused by emotional, physical or metabolic disruption. (Peckett et al., 2012)

Stress stimuli are consolidated in the paraventricular nucleus (PVN) of the hypothalamus, where the main source of corticotrophin-releasing hormone (CRH) is produced through the activation of CRH neurons (Aguilera and Liu, 2012). The neuronal projections of CRH neurons terminate in the regions of the arcuate nucleus (ARC) and median eminence (ME). Upon detection of a stress, CRH neurons release CRH into the hypophyseal portal system and bind to CRH receptors (CRH-R1) to stimulate the release of adrenocorticotropic hormone (ACTH) from POMC producing cells in the pituitary. Once ACTH is released into the bloodstream, plasma ACTH binds to its receptor on the adrenal cortex to stimulate the production and release of glucocorticoids (cortisol in humans, corticosterone (CORT) in rodents). Upon release, plasma CORT can exert its effects on various tissues to properly respond to the stimulus. CORT levels are controlled by negative feedback on the hypothalamus and the pituitary where CORT binds to its receptors sits own production (Figure 2). (Castro et al., 2011)



FIGURE 2. REGULATION OF HPA AXIS

Glucocorticoid inhibits the secretion of CRH from the PVN in the hypothalamus and ACTH from the pituitary, thereby downregulating its own production. Glucocorticoids also suppress the stress input by modulating the activation of immune cells and neuronal inputs from the brainstem and the limbic system that may maintain the HPA activation. Lastly, the production of β -endorphin from POMC neurons located in the ARC of the hypothalamus directly suppress the activation of CRH producing neurons in the PVN, thereby suppressing the activation of the HPA axis.

CORT elicits its actions by binding to Type I mineralocorticoid receptors (MR) or Type II glucocorticoid receptors (GR) on various cells throughout the body. CORT binds with high affinity to MR and these receptors are saturated in the presence of low CORT concentration. On the other hand, CORT binds to lower affinity to GR and requires high concentrations of CORT to be saturated. For this reason, it is believed that MR control the basal activity of the HPA axis, while GR control the activity of the HPA axis under stress response (Castro et al., 2011). The binding of CORT to GR modulates the HPA axis by suppressing the activation of cells involved in propagating the stress stimulus including the CRH neurons of the PVN as well as immune cells that produce inflammatory factors that could stimulate CRH release (Sliwowska et al. 2010).

The HPA axis of the developing fetus responds very similarly to stress. The HPA axis is functional by the second trimester and its activation has been shown to be necessary for proper organ maturation (Challis et al., 2001). Moreover, the second week of gestation represent the period of maximal HPA axis development in rodents (Weinberg et al., 2008). During this period, activation of the fetal HPA results in the secretion of CRH, ACTH and CORT in response to stimulation (Ng, 2000). On the other hand, a change in HPA axis occurs in the first postnatal week in rodents. A period of stress hyporesponsiveness (SHRP) takes place and stimulation of the HPA axis does not lead to increases in plasma CORT levels (Sapolsky and Meaney, 1986; Vazquez, 1994; Moriceau et al., 2010).

Interestingly, exposure to stressors during the second and/or third trimester has been shown to result in lifelong HPA programming regardless of basal differences in HPA response between these developmental periods (Sarkar et al., 2007; Weinberg et al., 2008; Hellemans et al., 2010; Koening et al., 2011; Bilbo, 2013). The HPA axis is especially sensitivity to ethanol induced reprogramming during the second and third trimester equivalent (Sarkar et al., 2007; Weinberg et al., 2008; Hellemans et al., 2010).

Dysfunctions in HPA pathways and/or regulation appear to be major contributors of HPA reprogramming following prenatal stress. Permanent alterations in CORT receptor expression has been shown to contribute to abnormal HPA response throughout the life of affected individuals (Koenig et al., 2011; Waffarn et al., 2012). More specifically, changes in GR receptors in the prefrontal cortex, hippocampus, amygdala, hypothalamus and pituitary contribute to delayed inhibition when GR expression is reduced and improper stress response when GR expression is increased (Seckl, 2004; Seckl and Meaney, 2004; Cottrell and Seckl, 2009).

Alternatively, changes in the function of cells that contribute to HPA axis activation and/or regulation may result in permanent HPA dysfunction following prenatal stress. Bendorphin (BEP) is an endogenous opioid cleavage product of the precursor protein proopiomelanocortin (POMC) (Figure 3). POMC is differentially cleaved depending on the stimulus and the tissue (Marinelli and Gianoulakis, 2004; Bicknell, 2008; Padilla et al., 2010). Upon HPA activation, CRH stimulates the preferential cleavage of ACTH from POMC expressing cells in the pituitary. ACTH is then secreted into the bloodstream to stimulate CORT production and secretion from the adrenal cortex, as previously discussed. In the ARC of the hypothalamus, where the highest density of POMC producing neurons is located, CRH stimulates the cleavage and release of BEP into the PVN. Upon release, BEP can act as a modulator of HPA axis by inhibiting CRH release from CRH-producing neurons in the PVN (Figure 2). Barfield et al., 2013



FIGURE 3. POMC PROCESSING

Schematic diagram of the POMC precursor molecule and the major peptide products that are derived from this precursor by endoproteolytic cleavage. Abbreviations: ACTH, adrenocorticotropic hormone; LPH, Lipotropin; β -END; β -Endorphin; N-POC, N-terminal POMC fragment; JP, Joining protein; CLIP, Corticotropin-like-intermediate lobe peptide; MSH, melanocyte-stimulating hormone; PC1/3 and PC2, Prohormone convertases 1/3 and 2. Adapted from Eves and Haycock, 2010

1.3 ETHANOL INDUCED APOPTOSIS OF HYPOTHALAMIC NEURONS

Using prenatal and postnatal rodents model (second and third human trimester equivalent, respectively), we have previously provided evidence that ethanol increases the apoptosis of hypothalamic cells, including BEP-expressing POMC neurons in the ARC (Chen et al., 2006; Sarkar et al., 2007; Kuhn and Sarkar, 2008; Boyadjieva et al., 2009). Moreover, we have demonstrated that the reduction of BEP expressing neurons following prenatal ethanol exposure contribute to HPA hyper response to immune challenge in adulthood (Boyadjieva et al., 2009).

It is well documented that ethanol exposure during fetal development increases apoptosis of neuronal cells (Ikonomidou et al., 2000; Sarkar et al., 2007; Boyadjieva et al., 2009; Gil-Mohapel et al., 2010; Brocardo et al., 2011; Kane et al., 2012). Ethanol induced neurotoxicity can occur through many mechanisms including the increase of oxidative stress and inflammatory markers that may lead to apoptotic signaling (Alfonso-Loeches and Guerri, 2011).

Oxidative stress refers to the imbalances between reactive oxygen species (ROS) and the levels of antioxidant that may help counteract their potential damage to cells and tissue. Although ROS are involved in important physiological processes, the increase of these species can lead to protein, lipids and DNA damage that may result in neuronal death. Endogenous enzymatic and non-enzymatic antioxidants serve to defend against damage by monitoring and inhibiting ROS production. (Brocardo et al., 2011)

The developing brain is particularly vulnerable to oxidative damage due to its high oxygen consumption rate and high content of ROS substrate. Moreover, the levels of antioxidants is as low as 41% that of adults for certain critical antioxidants such as glutathione peroxidase (GPX) (Brobardo et al., 2011). Amongst all antioxidants, the antioxidant enzymes superoxide dismustase (SOD), catalase (CAT) and GPX seem to be the most sensitive to the adverse effects ethanol exposure in the brain (Table 1) (Drever et al., 2012). It has been postulated that both decreases and increases in endogenous enzymatic enzymes reflect oxidative stress following ethanol exposure due to either increased neutralization or excess production of ROS (Haorah et al., 2008)

Enzymatic antioxidant	Cellular location	Substrate	Reaction
Superoxide dismutase (Mn/Cu/ZnSOD)	Mitochondrial matrix (MnSOD)	Superoxide ($O_2 \cdot)$	$O_2 \cdot \overline{} \to H_2 O_2$
Catalase	Peroxisomes Cytosol	Hydrogen peroxide (H ₂ O ₂)	$2H_2O_2 \rightarrow O_2 + H_2O$
Glutathione peroxidase (GPX) Peroxiredoxin I → VI (Prx)	Cýtosol Cytosol	Hydrogen peroxide (H ₂ O ₂) Hydrogen peroxide (H ₂ O ₂)	$\begin{array}{l} H_2O_2 + GSH \rightarrow GSSG + H_2O \\ H_2O_2 + TrxS_2 \rightarrow Trx(SH)_2 + H_2O \end{array}$

TABLE 1. DESCRIPTION OF ENDOGENOUS ANTIOXIDANT ENZYMES.Adapted from Gough and Cotter 2011

We have previously provided *in vitro* evidence that ethanol exposure increases oxidative stress in mixed hypothalamic neuronal culture as demonstrated by increased ROS levels and decreased activity of endogenous antioxidant enzymes (Boyadjieva and Sarkar, 2013a, 2013b). Moreover, we have recently found that the use of exogenous antioxidants can prevent ethanol induced neurotoxicity (Boyadjieva and Sarkar, unpublished). Interestingly, the neurotoxic effect of oxidative stress was shown to be primarily caused by ethanol induced microglial activation (Boyadjieva and Sarkar, 2010; Boyadjieva and Sarkar, 2013a, 2013b). In addition, ethanol induced microglial activation resulted in increased production and release of inflammatory factors such as macrophage inflammatory protein (MIP)1- α , MIP2, interleukin 6 (IL6) and TNF- α (Boyadjieva and Sarkar, 2010). Most importantly, ethanol induced neurotoxicity was shown to be almost entirely dependent on the production of TNF- α (Boyadjieva and Sarkar, 2010). Exogenous TNF-α potentiates the production of ROS upon ethanol exposure (Boyadjieva and Sarkar, unpublished). Moreover, the neutralization of TNF- α completely prevents the production of ROS and ethanol induced apoptotic neuronal death (Boyadjieva and Sarkar, 2010; Boyadjieva and Sarkar, unpublished). Taken together, this data suggest that ethanol induced microglial activation plays a critical role in the neurotoxic effect of ethanol exposure on hypothalamic neurons including BEP-expressing neurons.

1.4 MICROGLIA IN THE ADULT AND DEVELOPING BRAIN

Microglia are resident CNS innate immune cells that make up 5-20% of the cells in the adult brain, depending on the species (Polazzi and Monti, 2010). Microglia are myeloid lineage cells of hematopoietic origin that colonize the brain during the early embryonic period (Bodnar and Weinberg, 2013). With the guidance of region specific differentially expression of cytokines and chemokines (cytokine with chemoattractant properties), microglia cells migrate to their final destination and continue to proliferate and differentiate until early adolescent stage (Figure 4) (Schlegelmilch et al., 2011; Bilbo et al., 2012).



FIGURE 4. MATURATION AND PROLIFERATION OF MICROGLIA IN RODENTS.

Representation of microglia maturation, differentiation and proliferation from developmental period into adulthood. Abbreviations: E, Embryonic day; P, Postnatal day. Adaptation of Bilbo et al., 2012 and Harry, 2013

The morphology and function of immature microglia in the developing brain is distinct from those found in the adult brain (Streit, 2001; Dalmau et al., 2003; Deverman and Patterson, 2009; Bilbo, 2013). In the absence of a stimulus, mature microglia are in a ramified state where the cells survey and maintain the microenvironment and surrounding synapses (Polazzi and Monti, 2010; Kettenmann et al., 2013). The ramified microglia constitutively express various intracellular and transmembrane proteins and receptors that allow the cells to quickly detect changes in homeostasis and elicit a response (Kreutzberg, 1996; Takeuchi, 2010). The expression of these proteins also allows the cells to communicate with surrounding neurons and glia (Kettenmann et al., 2013). Upon detection of changes in homeostasis, microglia quickly become activated and undergoes distinct morphological alterations that are accompanied by the increase in expression of proteins associated with activation (Jonas et al., 2012). The activated microglia retracts its processes and enlarges its cell body while increasing its expression of surface molecules such as complement receptor 3 (CD11b and CD18), cytokine receptors and toll-like receptors (TLR) (Takeuchi et al., 2010). When the cell becomes fully activated and phagocytic, microglia acquires an amoeboid phenotype and can proliferate (Deverman and Patterson, 2009). In this state, the fully activated microglia increases its expression of major histocompatibility complex (MHC) molecules and can serve as an antigen presenting cell (Figure 5) (Town et al, 2005; Jones et al., 2012). The gradient of activation and expression of activation-associated proteins depend on various factors including the stimulus, the duration of the stimulus and the region of the CNS (Raivich et al, 1999; Town et al, 2005; Ransohoff and Perry, 2009).



FIGURE 5. SIMPLIFIED SCHEMATIC OF MICROGLIAL ACTIVATION

Ramified microglia constitutively express various proteins including IBA1 and CD11b. Upon stimulation, partially activated microglia increase the expression on these proteins, retracts its processes and enlarges its cell body. When the cell becomes fully activated, it can serve as an antigen presenting cell by expressing MHC I/II proteins. Abbreviations: IBA1, ionized calcium binding adaptor molecule 1; CD11b, cluster of differentiation molecule 11b; MHC, major histocompatibility complex.

On the other hand, immature microglia often express markers associated with activation in the adult brain, even in the absence of a stimulus. "Resting"/surveying microglia cells have an amoeboid phenotype, increased motility, and increased expression of surface markers often associated with full activation in mature microglia such as phagocytic marker CD68 (Figure 4) (Streit, 2001; Dalmau et al., 2003). Microglia play a crucial role in proper brain development. The presence of microglia is necessary for the removal of apoptotic bodies accumulated from normal apoptotic signaling associated with development (Streit, 2001). Microglia can also participate in the initiation of apoptotic signaling by releasing various factors such as pro-inflammatory cytokines and ROS that facilitate normal developmental cell death (Deverman and Patterson, 2009). In addition, microglia play a key role in synapse formation during development. Microglia have been shown to be necessary for synapse pruning and elimination in order to form proper functional neural circuits (Deverman and Patterson, 2009; Schafer et al., 2012; Schafer et al., 2013). Furthermore, the immature microglia also has the ability to detect changes in homeostasis and elicit a response. Their activation is also accompanied by an increase in receptor and protein expression, although the range of surface markers is more limited than that of their adult counterparts (Streit and Xue, 2009; Mizutani et al., 2011; Hickey et al., 2011; Harry, 2013).

Microglia are very sensitive to changes on the microenvironment and their threshold of activation is low. Their activation can lead to beneficial or detrimental effects on the CNS. The activated microglia produce and secrete various molecules including trophic factors, anti- and pro-inflammatory cytokines, chemokines, ROS, nucleic acids and proteases (Takeuchi, 2010). These factors facilitate the orchestration of immune signaling and initiate local microglia respond through the engulfment of pathogens or debris, repair of damage cells and synapses and recruitment other cells that may aid in restoring homeostasis (Pollazi and Monti, 2010).

The production and release of microglial inflammatory products is regulated by neuroimmune regulators (NIReg) produced by various cells in the CNS including neurons, astrocytes and microglia themselves (Hoarau et al., 2011). These include but are not limited to anti-inflammatory cytokines such as IL10, chemokines such as fractalkine (also known as CX3CL1) and complement regulators such as CD200 (Cordona et al., 2006; Yong, 2010; Badoer, 2010; Haorau et al., 2011; Shrivastana et al., 2012).

Due to their differential production of pro- and anti-inflammatory factors, it is believed that microglia have two main forms of activation: M1-like and M2-like macrophage phenotype. The M1-like macrophage phenotype is associated with the production of inflammatory factors whereas the M2-like macrophage phenotype is associated with the production of antiinflammatory factors (Yong, 2010; Tarassishin et al., 2011; Bilbo, 2013; Marshall et al., 2013). These two states of alternate activation possess similar morphological phenotypes which make them difficult to distinguish. Therefore functional analysis and quantification of their products have been deemed more reliable than the assessment of activation solely based on morphological changes (Fumagalli et al., 2011; Schwarz et al., 2011; Marshall et al., 2013).

1.5 ETHANOL INDUCED MICROGLIAL ACTIVATION

Microglial activation has been shown to contribute to neurodegeneration when the productions of its inflammatory factors are not regulated. This effect has been observed across numerous neurodegenerative diseases including alcohol-related pathologies (Crews et al., 2006; Boyadjieva and Sarkar, 2010; Gao et al, 2011; Zhao et al., 2013). Post mortem brain analysis of alcoholics and moderate alcohol drinkers reveals increased microglia activation and chronic NFkB inflammatory signaling (He and Crews, 2008; Crews et al., 2011). Furthermore, intermittent and chronic alcohol exposure increases neurodegeneration and activation of microglia in adolescent and adult rats (Alfonso-Loeches and Guerri, 2011; Qin and Crews, 2012; Zhao et al. 2013). The use of minocycline (a second generation tetracycline known to suppress microglia inflammatory signaling) prevents ethanol induced inflammation and neurodegeneration in rodents following chronic ethanol exposure (Qin and Crews, 2013). These results provide evidence for the involvement of microglia in ethanol induced neurodegeneration in the mature brain.

It is believed that ethanol induced neurodegeneration during the developmental period may entail similar mechanisms. As previously discussed, we have observed increased neurodegeneration of hypothalamic neurons following exposure to ethanol conditioned microglia media (Boyadjieva and Sarkar, 2010). Several *in vivo* studies have also reported correlative evidence of microglia activation, elevation of pro-inflammatory factors and increased vulnerability to ethanol induced neurodegeneration following prenatal or neonatal ethanol exposure (Watari et al., 2006; Kane et al., 2011; Saito et al., 2012). These studies suggest that microglia activation during the developmental period increases the brain's vulnerability to neurodegeneration possibly due to increased inflammatory signaling.

1.6 STRESS INDUCED MICROGLIA PROGRAMING

In the healthy CNS, microglia quickly detect a stimulus, becomes activated and elicit the proper response. Once the stimulus is removed and homeostasis is restored, microglia revert to their inactive ramified state and return to surveillance duties (Jones et al., 2012). The deactivation of microglia is also associated with the reduction of the surface markers that were increased during activation (Raivich et al., 1999). Recent evidence demonstrate that complete microglial deactivation may not occur following ethanol exposure.

Microglia remain activated for extended amounts of time in the brain of alcoholics, even during abstinence (Crews et al., 2011). This provides evidence that chronic ethanol exposure can permanently activate microglia. This effect also seems to be present following intermittent ethanol exposure. It has recently been shown that microglia remain activated for a few days following binge-like ethanol exposures in adult rats (Zhao et al., 2013). Interestingly, adolescent rats exposed to a single four day binge-like drinking episode display persistent microglia activation of at least 30 days (McClain et al. 2011). This suggest that ethanol induced microglial activation is more vulnerable to persistent activation in the developing brain compared to the adult brain. In addition, ethanol primes microglia and exaggerate their response to subsequent stimuli. Ethanol exposure followed by the administration of the TLR3 agonist poly I:C increased microglial production of pro-inflammatory cytokines (Qin and Crews, 2012). It has been proposed that the ethanol induced priming of microglia to subsequent stimuli result from incomplete activation following ethanol exposure (McClain et al., 2011; Qin and Crews, 2012; Zhao et al., 2013).

Microglial responses are also exaggerated following neonatal stress. Exposure to stressors, including ethanol, during the early developmental period results in microglial activation (Kane et al., 2011; Bilbo et al., 2012). Moreover, Williamson et al (2011) have demonstrated that a single exposure to Escherichia coli (E. Coli) activate microglia in rat neonates, and causes long term alterations in microglial response and behavior in adulthood. However, only minimal differences in microglia were observed between E. coli exposed animals and controls at baseline in adulthood, suggesting that alterations in microglial responses following neonatal activation may be due to reprogramming rather than persistent activation (Williamson et al., 2011; Bilbo, 2013). No previous investigation on the long term effects of ethanol induced microglial activation during the neonatal period have been reported.

Correlative data have shown that increased CNS inflammation during early development increases the risk of developing neurologic and neuropsychiatric disease associated with HPA dysfunction (Hagberg et al., 2012; Beumer et al., 2012; Bilbo, 2013). In theory, any stressors that activate microglia and thereby increased inflammatory signaling during developing may contribute to inflammation induced mental disorders. Moreover, if neonatal stress programs microglia to respond abnormally to subsequent stimuli in adulthood, the amplified inflammatory signaling that may result following microglia activation may contribute to increased HPA activation. In this study, we investigate the role of microglia in ethanol induced alterations in HPA axis response observed in fetal alcohol exposure model.

HYPOTHESIS

To determine whether ethanol induced microglia activation during the neonatal period contribute to the hyper stress response observed in fetal alcohol exposure model.

Aims of the study:

- Aim 1. To determine if neonatal ethanol exposure induces microglial activation and production of inflammatory factors
- Aim 2. To determine if neonatal ethanol exposure programs microglia to respond differently to subsequent stressors in adulthood
- Aim 3. To determine the role of neonatal ethanol induced microglial activation in the neurotoxicity of β -endorphin neurons

CHAPTER 2

2.1 INTRODUCTION

Alcohol exposure leads to the production of various factors including cytokines, chemokines and reactive oxygen species (Cui et al, 2013; Qin et al, 2012; Boyadjieva and Sarkar, 2010). Its chronic use has been shown to have detrimental effects on the peripheral immune system leading to increase incidences of infections and diseases such as cancer and cardiovascular diseases (Zhang et al, 2012; Poli et al, 2013). Chronic alcohol use has also been associated with enhanced cytokine production and oxidative stress in the CNS. In the adult brain, ethanol's effects partially occur through the activation of the CNS innate immune system, mainly comprised of microglia, which leads to enhanced neuroinflammatory signaling (Cui et al, 2013; Boyadjieva et al, 2010).

In the absence of a stimulus, microglia remain in a "resting" ramified state where the cell surveys its surrounding and maintains neighboring cells. When changes in homeostasis are detected, microglia rapidly become activated and secrete a variety of factors including neurotrophic growth factors, cytokines and chemokines. Their activation is associated with dramatic morphological changes as well as a marked increase in expression of surface proteins and receptors. This activation can either be neurotrophic or neurotoxic, depending on the stimulus and the response of the microglia (Badoer, 2010; Kane et al, 2012).

Ethanol affects microglia responses differently depending on the dose and duration of alcohol exposure, method of administration and brain region (Kane et al, 2012; Kane et al, 2013). Interestingly, their response to the same stimulus can also vary depending on the age of the individual (Kane et al, 2012; Kane et al., 2013). Various aging studies demonstrate that microglia sensitivity and responses normally change throughout the lifetime of individuals (Kane et al, 2013; Bilbo et al, 2013; Bachstetter et al, 2011; Nije et al. 2012; Streit and Xue, 2009). Microglia seem to be most stable in the adult brain although their reactivity increases during late stages of adulthood as demonstrated by studies comparing young versus aged brains (Bachstetter et al,

2011; Nije et al, 2012; Harry, 2013). Immature microglia in the developing brain also differ from the mature microglia found in the adult CNS. In addition to morphological differences, immature microglia seem to be more sensitive to stimuli compared to mature microglia (Bilbo et al, 2013; Harry, 2013).

It has been shown that ethanol exposure induces microglial activation in various brain regions in adults and this effect has been supported by many *in vitro* studies. Most of what is known about ethanol induced microglial activation during development comes from microglia in culture (Boyadjieva 2010, 2012; Benjamins et al., 2011). Some studies have demonstrated that ethanol induced activation of microglia in the later stages of brain development results in neuroinflammatory signaling which may contribute to the neurodegeneration observed in fetal alcohol exposed brain (Kane et al, 2011). However, little is known about the effect of ethanol exposure on hypothalamic microglia throughout the third trimester *in vivo*. Here, we examined the effect of ethanol exposure on microglial activation in the hypothalamus during the third trimester equivalent in rodents.

2.2 MATERIALS AND METHODS

Animal Use

Sprague-Dawley females and males were purchased from Charles River and mated. The rats were housed in 12h light/ 12h dark cycles (lights on at 7:00 am and off 7:00 pm) on a constant temperature (22°C) throughout the study. Fetal brains were used for primary microglia culture and neonates were used for postnatal ethanol treatment. Animal surgery and care were performed in accordance with institutional guidelines and complied with the National Institutes of Health policy. The animal protocol (99-005) was approved by Rutgers Animal Care and Facilities Committee.

Fetal Alcohol Exposure Model

One day after birth, neonates were divided into one of four groups; Ad libitum (AD), Pair-fed (PF), Alcohol-fed (AF) and Alcohol-Fed + Minocycline (AF+M). The AD group were only nursed by the mother. The PF, AF and AF+M neonates were fed twice daily by intubation with a milk formula (PF) or with milk formula containing alcohol (11.34% v/v) (AF and AF+M) in addition to nursing for up to five days with two hours between feedings. The AF+M group received a minocycline pre-treatment one hour prior to the first feeding on each day by subcutaneous injection of minocycline solution ($45\mu g/kg$ bodyweight). Each brain was collected one hour after last feeding and immediately frozen and stored at -80°C.

Microglia Culture

Microglial cells were prepared from E19-PND1 rat hypothalamic tissue using a method modified previously described (McCarthy and de Vellis, 1980). Hypothalami were dissociated by passing through a 18G needle and then through a 70-lm Nitrex mesh and plated at 2 x 10^5 cells/cm². Cultures were fed every 4 days with DMEM/MEM/Hams 12 (DMEMF12) in a 4:5:1 ratio with 10% FCS. On day 14, the culture was shaken on a rotary shaker at 800 rpm for 2 hour (Frei et al., 1987). The suspended cells were plated on uncoated T25 flasks and incubated at 37°C and fed with DMEMF12 to develop the microglia culture. After an additional 7 days, cultures were shaken on a rotary shaker for 2 hours and suspended cells were plated on 24 well plates and maintained in DMEMF12 in 24-well plates (1 x 10^5) for experiments.

Two days after isolation, the cells were challenged for 24 or 48 hours with ethanol at a concentration of 50 mM (0.3% v/v), 100 mM (0.6% v/v) or vehicle. LPS (10 ng/mL) challenge for 6 hours was also used as a positive control for microglia activation. After challenge, the medium from cultured microglial cells was collected and centrifuged. The supernatants were used for quantification of pro-inflammatory cytokines.

Cytokine Quantification in Microglia Media Culture

The protein levels of TNF- α , IL1 β and IL6 in the medium were quantified by enzymelinked immunosorbent assay (ELISA) methods from commercially available kits and were conducted according to the manufacturer's protocol (Enzo Life Sciences, Cat# 900-086A and Cat# 900-131; Life Technologies, Cat# KRC0061, respectively).

Cytokine Protein Assay for Mediobasal Hypothalamic Lysate Neonatal Pups.

The arcuate nucleus was removed by sectioning off the anterior portion of the mediobasal hypothalamus up to the end of the optic chiasm and using the tissue posterior of the optic chiasm until the end of the mediobasal hypothalamus. The most ventral part was then micropunched using 200 μ L calibrated micropipets. The tissue was then homogenized in 150 μ L of 1X PBS with protease inhibitor.

The protein levels of TNF- α , IL1 β , IL6, MCP1, CX3CL1 and IL10 were quantified by enzyme-linked immunosorbent assay (ELISA) methods from commercially available kits. IL1 β , IL6, MCP1, CX3CL1 and IL10 assay were conducted according to the manufacturer's protocol (Enzo Life Sciences, Cat# 900-131; Life Technologies, Cat# KRC0061; Abcam, Cat# Ab100778, Cat#Ab100761 and Cat#100765, respectively). TNF- α assay was conducted according to manufacturer's protocol with the minor modification of 2 hours of sample incubation en lieu of the recommended 1 hour (Enzo Life Sciences; Cat# 900-086A). The quantified values were normalized by total protein concentration determined by Bradford Assay (Bio-Rad Laboratories, Cat# 500-0201) for each sample.

Immunofluorescence and Immunohistochemistry

Serial coronal sections of frozen brains were made using *Leica* cryostat at 20 μ m in thickness from stereotaxic plates 17 to plate 23 (approximately Bregma -1.4 mm to -4.5 mm) spanning from the paraventricular nucleus (PVN) to the arcuate nucleus (ARC) area. Three plate-

equivalent sections were placed on each slide contained either one AD section, one PF section and one AF section or three sections of AF+M.

A total of 3 sections per brain were stained for all proteins detected within the PVN and 10 sections per brain were used for detection within the ARC. All primary antibodies were incubated overnight. Total microglia cell population was detected by immunofluorescence using a rabbit polyclonal antibody for ionized binding calcium adaptor molecule 1 (IBA1) (1:400, Wako, Cat# 019-19741). IBA1 is a 17-kDa protein constitutively expressed in microglia. AlexaFluor 488 donkey anti-rabbit and AlexaFluor 488 donkey anti-goat (1:1000, Molecular Probes, Cat#A21206 and A11055, respectively) were used as secondary antibodies for immunoflorescence. After staining, the slides were mounted in DAPI (Vector Laboratories, Cat# H-1200) and covered with a 1mm coverslip.

Activated microglia were detected by immunohistochemistry using the mouse monoclonal CD11b (clone OX42) (1:50, Abd Serotec, Cat# MCA275). CD11b forms part of the complement receptor 3 and is expressed on the surface of microglia. Its expression increases markedly upon activation of microglia (Kim et al., 2000; Tsuda et al., 2003). After overnight primary antibody incubation, sections were incubated with peroxidase-coupled anti-mouse Ig ImmPRESS reagent (Vector Laboratories, Cat# MP7402). Antigen localization was achieved by using the 3,3'-diaminobenzidine-peroxidase reaction and sections were then dehydrated and cover with a 1mm coverslip.

Pictures were taken using inverted microscope (Nikon EZ-C1 build 770, Gold Version). IBA1 and CD11b expressing cells were counted to the left and right of the third ventricle covering a total area of 1mm² for each section of the PVN and ARC region under 20X magnification.

Antioxidant and Reactive Oxygen Species Quantification
The arcuate nucleus was removed by sectioning off the anterior portion of the mediobasal hypothalamus up to the end of the optic chiasm and using the tissue posterior of the optic chiasm until the end of the mediobasal hypothalamus. The most ventral part was then micropunched using 200 μ L calibrated micropipets. For detection of antioxidant level, the tissue was homogenized in 150 μ L of 1X PBS with protease inhibitor. Total antioxidant capacity was quantified using antioxidant assay kit according to manufacturer's protocol (Sigma-Aldrich; Cat# CS0790). For quantification of total reactive oxygen species (ROS), another set of samples were micropunched and gently homogenized in 100 μ L of 10mM solution of carboxy-H2-DCFH-DA (DCFH-DA) (Molecular Probes, Cat# D23107) and incubated at 37°C for 45 min in dark. After incubation, the samples were centrifuged for 5 min at 250g. The supernatant was removed; cell pellets were washed by re-suspending in PBS, centrifuged again for 5 min at 250g. The cells were then re-suspended in 100 μ L of PBS. Fluorescence was read at 485 nm excitation and 535 nm emission on a fluorescence plate reader.

Quantitative Real-Time PCR of Antioxidant Enzymes

Mediobasal hypothalamic tissue was collected as described above. The tissue was then homogenized in 200 µL of Trizol and RNA isolation was completed using PureLink RNA Isolation kit according to manufacturer's protocol (Life Technologies). cDNA conversion was performed using high capacity cDNA reverse transcriptase kit (Applied Biosystems). Yield cDNA was used to quantify GPX4, CAT1, SOD1 and SOD2 mRNA expression using Taqman Gene Expression Assay probes (Applied Biosystems (ABI); Table 2). A standard curve was included on each plate. Relative expression was determined and normalized against GAPDH housekeeping gene.

GENE	ABI PROBES
SUPEROXIDE DISMUTASE 1 (SOD 1)	Rn00566938_m1
SUPEROXIDE DISMUTASE 2 (SOD 2)	Rn99999088_g1
CATALASE (CAT)	Rn00560930_m1
GLUTATHIONE PEROSIDASE 4 (GPX4)	Rn00820818_g1
GLYCERALDEHYDE 3- PHOSHATE DEHYDROGENASE (GAPDH)	Rn99999916_m1

TABLE 2. LIST OF SELECTED ANTIOXIDANT ENZYMES GENES

Statistical Analysis

Graph Pad Prism version 4 software was used for statistical analysis of data. Quantitative results are indicated as mean \pm SEM. The significance between treatment and controls was assessed using a two-way ANOVA between treatment and days of exposure. Post hoc analysis using Bonferroni post test was performed. P < 0.05 was considered significant.

2.3 RESULTS

Ethanol significantly increased microglial activation in the PVN and ARC of AF neonates compared to controls as quantified by increased number of CD11b⁺ expressing cells. Increased microglial activation in AF pups occurred as early as the first day of exposure in the PVN and persisted even after five days of exposure (Figure 6A & B). Expression of CD11b⁺ cells in the ARC did not differ in AF pups compared to controls until five days of ethanol exposure (Figure 7A & B). This increase in microglial activation in ethanol exposed pups was prevented by minocycline pre-treatment in both PVN (Figure 6A & C) and ARC (Figure 7A & C).



B.





DAYS OF EXPOSURE

5 DAYS OF EXPOSURE

Microglial activation was quantified by detection of CD11b⁺ immnoreactive cells in the paraventricular nucleus (PVN). (A) Showing the representative immunohistochemistry pictures (10X) of CD11b+ in the PVN of AD, PF, AF and AF+M rat pups after 5 days of exposure. (B) quantification of CD11b+ cell numbers in PVN for 1-5 days of exposure and (C) after 5 days of exposure. Data are mean \pm SEM of 5 independent observations. Two-way ANOVA followed by Bonferroni post-test revealed main effect between treatments (P = 0.0016) and significant difference between AD and AF pups throughout days of exposure (* P<0.05). One-way ANOVA followed by Bonferroni post-test revealed significant differences between AD and AF pups as well as minocycline pre-treated AF+M and AF pups after 5 days of exposure (c P<0.05).



FIGURE 7. ETHANOL EXPOSURE INCREASES MICROGLIAL ACTIVATION IN THE ARCUATE NUCLEUS IN NEONATES.

Microglial activation was quantified by detection of $CD11b^+$ immnoreactive cells in the arcuate nucleus (ARC). (A) Showing the representative immunohistochemistry pictures (10X) of CD11b+ in the ARC of AD, PF, AF and AF+M rat pups after 5 days of exposure. (B) Quantification of CD11b+ cell numbers in ARC for 1-5 days of exposure and (C) after 5 days of exposure. Data are mean \pm SEM of 5 independent observations. Two-way ANOVA followed by Bonferroni post-test revealed main effect between treatments (P<0.0001) and AF significantly different from AD pups (*** P<0.001) and PF (b" P<0.001) after 5 days of exposure. One-way ANOVA followed by

Bonferroni post-test revealed AF significantly different from AD, PF pups and minocycline pretreated AF+M (*** P<0.001, b" P<0.001 and c' P<0.01 respectively) after 5 days of exposure.

In order to determine if the drastic increase in number of activated microglia in the ARC of AF neonates was a result of increased microglia population from recruitment or proliferation, the total microglia cell number was quantified. Although an increase in microglia number was initially observed, there were minimal differences in total microglia population between groups by 5 days of ethanol exposure (Figure 8).





FIGURE 8. ETHANOL EXPOSURE DOES NOT PERSISTENTLY INCREASE MICROGLIA POPULATION IN THE ARCUATE NUCLEUS IN NEONATES.

Total microglia population was quantified by detection of $IBA1^+$ immnoreactive cells in the arcuate nucleus (ARC). (A) Showing the representative immunofluorescence pictures (10X) of IBA1+ in the ARC of AD, PF, AF rat pups after 5 days of exposure. (B) Quantification of IBA1+ cell numbers in ARC for 1-5 days of exposure. Data are mean \pm SEM of 6 independent observations. Two-way ANOVA followed by Bonferroni post-test revealed main effects of treatments and days of exposure (P<0.0001). AD pups (* P<0.05) and PF (b P<0.05 and b' P<0.01) significantly different from AF pups.

In order to confirm microglia reactivity to ethanol, primary MBH microglia were exposed to ethanol at 50 and 100 mM for a period of 24 or 48 hours. Ethanol induced the release of proinflammatory cytokines in a time and concentration dependent manner (Figure 9). The *in vivo* analysis of pro-inflammatory cytokines revealed that ethanol exposure also increased proinflammatory cytokines in the MBH of neonates in a time dependent manner. TNF- α , IL6 and MCP1 all increased in response to ethanol exposure compared to controls (Figure 10). Ethanol induced increases in pro-inflammatory cytokine expression was prevented by minocycline pretreatment in alcohol exposed neonates (Figure 10) however minocycline pre-treatment had no effect on control animals after 1 day of exposure (Supplemental Figure 1). Ethanol exposure also altered the expression of microglia modulator CX3CL1 and the anti-inflammatory cytokine IL10 in a time dependent manner (Figure 11). The reduction in the CX3CL1 correlated with the increase in many pro-inflammatory cytokines.



FIGURE 9. ETHANOL INDUCED MICROGLIAL ACTIVATION RESULTS IN PRO-INFLAMMATORY CYTOKINE RELEASE *IN VITRO*.

Pro-inflammatory cytokine release was quantified in the microglia media after ethanol exposure by ELISA. (A) TNF- α , (B) IL6 and (C) IL1- β cytokine levels in media after exposure. Data are mean \pm SEM of 5 independent observations. One-way ANOVA followed by Bonferroni post-test revealed main effects of treatments. *P< 0.05 significantly different between groups.



FIGURE 10. ETHANOL INDUCES THE RELEASE OF PRO-INFLAMMATORY CYTOKINES IN THE MEDIOBASAL HYPOTHALAMUS OF NEONATES.

(A) TNF- α , (B) IL6, (C) MCP1 and (D) IL1- β cytokines levels in protein lysate of MBH tissue in rat pups after exposure. All values were normalized against mean AD values. Data are mean \pm SEM of 5 independent observations. Two-way ANOVA followed by Bonferroni post-test revealed main effect of interaction between treatments and days of exposure for TNF- α ; main effect of treatment in IL6; main effects of treatment, days of exposure and interaction for MCP1(P<0.0001). *P<0.05, **P<0.01 and ***P<0.001 significantly different between AD and AF pups. b P<0.05, b' P<0.01 and b'' P<0.001 significantly different between PF and AF pups. c P<0.05 and c'' P<0.001 significantly different between AF+M and AF pups.



FIGURE 11. ETHANOL ALTERS THE EXPRESSION OF MICROGLIA MODULATORS IN THE MEDIOBASAL HYPOTHALAMUS OF NEONATES.

(A) CX3CL1 chemokine and (B) IL10 cytokine levels in protein lysate of MBH tissue in rat pups after exposure. All values were normalized against mean AD values. Data are mean \pm SEM of 5 independent observations. Two-way ANOVA followed by Bonferroni post-test revealed main effects of treatments and days of exposure for CX3CL1 and main effects of days of exposure and interaction between treatments and days of exposure in IL10 (P<0.0001). *P<0.05, **P<0.01 and ***P<0.001 significantly different between AD and AF pups. b" P<0.001significantly different between PF and AF pups.

Alcohol has been shown to induce oxidative stress in the adult and developing brain (Boyadjieva and Sarkar, 2013; Brocardo et al., 2011; Haorah et al., 2008), therefore we sought out to determine if ethanol exposure increased oxidative markers in neonates. Unexpectedly, we detected decreased total ROS in the hypothalamus of alcohol exposed neonates (Figure 12A). Moreover, we detected alterations in total antioxidant capacity throughout the days of exposure in AF neonates (Figure 12B).

In order to determine if the increase in total antioxidant capacity observed after acute ethanol exposure resulted from increases in transcription of antioxidant enzymes, we quantified the mRNA expression levels of the antioxidant enzymes most commonly affected by ethanol in the brain. We found no significant increase in mRNA expression of antioxidant enzymes in the hypothalamus following neonatal ethanol exposure (Figure 13).



FIGURE 12. ETHANOL ALTERS TOTAL ANTIOXIDANT CAPACITY IN THE MEDIOBASAL HYPOTHALAMUS OF NEONATES.

(A) Total Reactive Oxidative Stress (ROS) and (B) Total antioxidant capacity of MBH tissue in rat pups after exposure. Data are mean \pm SEM of 5 independent observations. Two-way ANOVA followed by Bonferroni post-test revealed main effects of treatments for Total ROS; main effect of interaction between treatment and days of exposure for Total Antioxidant Capacity (P<0.001). *P<0.05, **P<0.01 and ***P<0.001 significantly different between groups.



FIGURE 13. ETHANOL DOES NOT INCREASE THE TRANSCRIPTION OF ANTIOXIDANT ENZYMES IN THE MEDIOBASAL HYPOTHALAMUS OF NEONATES.

(A) Glutathione Perozidase 4 (GPX4), (B) Catalase 1 (CAT1), (C) Superoxide Dismutase (SOD1) and (D) SOD2 of MBH tissue in rat pups after exposure. Data are mean \pm SEM of 5 independent observations.

2.4 DISCUSSION

We show for the first time the progressive increase in microglial activation in the mediobasal hypothalamus of neonates in response to ethanol exposure. Here, we provide evidence that the number of ethanol induced activated microglia gradually increased *in vivo* as demonstrated by the augmentation of CD11b⁺ expressing cells in the PVN and the ARC of AF neonates compared to controls. Surprisingly, the increase of CD11b⁺ expressing cells depended on the length of exposure and seemed to differ depending on the region of the hypothalamus. Daily minocycline pre-treatment prevented the augmentation of activated microglia in ethanol exposed neonates. Ethanol exposure during neonatal period transiently altered pro-inflammatory cytokine expression in the mediobasal hypothalamus (MBH) of neonates as demonstrated by the variations in TNF- α , IL6, and MCP1 expression throughout exposure. In addition, ethanol differentially altered the expression of the anti-inflammatory cytokine IL10 in a time dependent manner. Interestingly, IL10 has been shown to vary depending on the state of microglial activation and is often associated with M1-like alternative activation (Bilbo et al., 2012; 2013; Marshall et al., 2013).

This suggests that ethanol's effects on hypothalamic microglia vary depending on the length of exposure, and may result in alternative activation states which respond to the stimulus differently by producing distinct factors. This theory would support other studies showing length of exposure is an important factor in determining the response of immature microglia to a stimulus (Alfonso-Loeches et al, 2011; Gao et al, 2010; Blandino et al, 2009).

We show for the first time that ethanol exposure during the third trimester equivalent temporally increases the expression the microglia modulator CX3CL1, also known as fractalkine. The chemokine CX3CL1 binds with high affinity to a single receptor, CX3CR1 (Ransohoff, 2011; Adler and Roger, 2005). CX3CL1 is almost exclusively produced by neurons and its receptor is mainly located on the surface of microglia in the CNS (Ransohoff, 2011; Adler and Roger, 2005). Although CX3CL1 is necessary for proper synaptic formation during development,

it expression is generally low throughout the lifetime of the healthy individuals and only increases after neuronal damage or inflammation (Zujovic et al., 2000; Barrientos et al., 2012). Damaged or injured neurons generally produce and secrete CX3CL1 to communicate with microglia and send "find me and help me" signals while suppressing the "kill me or eat me" signals (Deverman and Patterson, 2009; Ransohoff, 2011). Once the neuron becomes too damaged for repair, CX3CL1-CX3CR1 signaling is reduced and other factors such as pro-inflammatory cytokines and ROS increase "eat me" or "kill me" signals to allow microglia cells to amplify apoptotic signaling and engulf apoptotic bodies and debris (Cardona et al., 2006; Hoarau et al., 2011; Jaerve and Müller, 2012).

Alterations in CX3CL1 signaling have been recently associated with many neurodegenerative diseases including Parkinson's disease, Alzheimer's disease and FASD (Pabon et al., 2011; Lee et al., 2010; Cho et al., 2011; Cribbs et al., 2012; Roberson et al, 2011). CX3CL1's primary functions include chemotactic, anti-inflammatory and anti-apoptotic signaling (White and Greaves, 2011). These pathways are generally modulated by CX3CL1 through the downregulation of microglial activation and reduction their inflammatory signaling (Zujovic et al., 2000; White and Greaves, 2011). Our data suggest that CX3CL1 may play a role in the differential expression of pro-inflammatory cytokines in the hypothalamus and may even contribute to the delay in increase CD11b⁺ expressing cells after ethanol exposure. Taken together, our data suggests that ethanol induced microglial activation may significantly increase following prolonged ethanol exposure partially due to the reduction of microglia modulators such as CX3CL1.



SUPPLEMENTAL FIGURE 1. EFFECT OF MINOCYCLINE PRE-TREATMENT ON PRO-INFLAMMATORY CYTOKINES IN CONTROL NEONATES.

(A) TNF- α , (B) IL6 and (C) MCP1 cytokines levels in protein lysate of MBH tissue in rat pups after minocycline treatment. All values were normalized against mean AD values. Data are mean \pm SEM of 5 independent observations. Two-way ANOVA followed by Bonferroni post-test revealed main effect days of exposure for IL6 and MCP1 (P<0.01 and P<0.001, respectively). *P<0.05 significantly different from AD.

CHAPTER 3

3.1 INTRODUCTION

Ethanol induced disruptions in microglia sensitivity and signaling have been linked to many diseases including neuro-cognitive deficits (Crews et al., 2011) and anxiety like behaviors (Bilbo and Schwarz, 2009; Schwarz and Bilbo, 2013; Zhao et al., 2013). There is emerging evidence that ethanol induced activated microglia may not completely revert to an inactive state in the absence of the stimulus. McClain and colleagues (2011) previously showed that a single four day ethanol binge increases microglial activation in adolescent male rats and this activation persisted even after 30 days of abstinence. Moreover, chronic ethanol exposure enhances microglial activation and responses to subsequent stimuli in the absence of ethanol in adult brain (Qin and Crews, 2012). Taken together, these studies suggest that ethanol exposure may permanently alter microglial activation threshold, possibly by maintaining partial activation even in the absence of ethanol.

This may be particularly important in fetal alcohol spectrum disorder (FASD). It is well documented that FASD patients are more susceptible to mental disorders including depression and anxiety (Hellemans et al., 2008). Ethanol exposure during the developmental period results in lifelong changes in innate immunity and neuroendocrine responses (Sarkar et al., 2010; Weinberg et al., 2008; Hellemans et al., 2008; Sliwowska et al., 2006). Microglia, which are long lived self-replenishing CNS immune cells, can initiate and maintain stress activation by releasing pro-inflammatory cytokines. Alterations in microglial responses to stress stimuli may contribute to the hyper response to stressors observed in FASD patients, possibly through the increase expression of pro-inflammatory cytokines.

It has recently become evident that early-life immune activation can result in reprogramming of microglia. Early life infection studies demonstrate that CNS innate immune cells, particularly microglia, are highly vulnerable to reprogramming during the third trimester equivalent in rodents (Bilbo, 2013; Spencer et al., 2010). Williamson and colleagues (2011)

demonstrated that a single exposure to E. coli during the developmental period, specifically PD4, results in hyper response to psychological stressors in adulthood. Most importantly, they were able to show that this increase in stress response was partially due to increased cytokine production from CD11b⁺ expressing microglia and not GFAP expressing astrocytes (Williamson et al., 2011). Although these studies suggest that altered microglial activation may contribute to enhanced HPA axis response, it is unknown whether ethanol induced microglial activation during third trimester equivalent contributes to HPA hyper response in adulthood in a similar fashion. Here, we examined the effects of neonatal ethanol induced microglial activation on the stress response of adult males.

3.2 MATERIALS AND METHODS

Animal Use

Postnatal Alcohol Model and Bromodeoxyuridine Treatment

Sprague-Dawley females and males were purchased from Charles River and mated in house. The rats were housed in 12h light/ 12h dark cycles (lights on at 7:00 am and off 7:00 pm) on a constant temperature (22°C) throughout the study. One day after birth, male neonates were divided into one of four groups; Ad libitum (AD), Pair-fed (PF) and Alcohol-fed (AF) The AD group were only nursed by the mother. The PF and AF neonates were fed twice daily by intubation with a milk formula (PF) or with milk formula containing alcohol (11.34% v/v) (AF) in addition to nursing for up to five days with two hours between feedings. All pups received a daily subcutaneous injection bromodeoxyuridine (BrdU; Sigma-Aldrich, Cat#B5002) (50mg/kg bodyweight) at the time of the first feeding. BrdU incorporates into newly synthesized DNA and its detection indicates that the cell was actively replicating its DNA during exposure of BrdU, an event that occurs during cell division. For this reason, BrdU incorporation is used for detection of cell proliferation.

The number of neonates was normalized to six pups per dams and each litter contained different treatment groups to reduce litter effects. After 5 days of ethanol and BRDU treatment, male neonates were kept with dam until PD 21 and were then weaned and housed in groups of two or three animals per cages and were left to grow into adulthood. At PD90, adult males were sacrificed and brains were collected and immediately frozen and stored at -80°C. Animal surgery and care were performed in accordance with institutional guidelines and complied with the National Institutes of Health policy. The animal protocol (99-005) was approved by Rutgers Animal Care and Facilities Committee.

Postnatal Ethanol Treatment and Restraint Stress

Male neonates were segregated into AD, PF and AF groups and treated as described above. At PD 90, basal tail blood was collected at 10:00 AM in adult males. After basal blood collection, animals were immediately placed into restraint tubes and subjected to 60 minutes of restraint stress followed by a recovery period where the animals were returned into their homecages. Animals were habituated with homecages for three days before stress experiments. Tail blood was collected following 15, 30 and 60 min of restraint and 30, 60 and 120 min after restraint in ethylenediamintetracetic acid (EDTA) (62 mg/mL; EDTA; Sigma-Aldrich) containing tubes. Each brain was collected after 120 min post restraint and immediately frozen and stored at -80°C.

Postnatal Alcohol Model and Lipopolysaccharide (LPS) treatment

Sprague-Dawley females and males were purchased from Charles River and mated in house. The rats were housed in 12h light/ 12h dark cycles (lights on 7:00 am and off 7:00 pm) on a constant temperature (22°C) throughout the study. One day after birth, male neonates were divided into one of four groups; Ad libitum (AD), Pair-fed (PF), Alcohol-fed (AF) and Alcohol-Fed + Minocycline (AF+M). The AD group was only nursed by the mother. The PF, AF and

AF+M neonates were fed twice daily by intubation with a milk formula (PF) or with milk formula containing alcohol (11.34% v/v) (AF and AF+M) in addition to nursing for up to five days with two hours between feedings. The AF+M group received a minocycline pre-treatment one hour prior to the first feeding on each day by subcutaneous injection of minocycline solution ($45\mu g/kg$ bodyweight). The number of neonates was normalized to six pups per dams and each litter contained different treatment groups to reduce litter effects. After 5 days of ethanol treatment, male neonates were kept with dam until PD 21 and were then weaned and housed in groups of two or three animals per cages and were left to grow into adulthood.

At PD90, adult males received a single LPS dose (100ug/kg bodyweight i.p.; Sigma-Aldrich) at 10:00 am. Tail blood was collected for basal hormone quantification prior to LPS exposure and trunk blood was collected two hours after LPS exposure in EDTA containing tubes. Brains were collected and immediately frozen and stored at -80°C. Animal surgery and care were performed in accordance with institutional guidelines and complied with the National Institutes of Health policy. The animal protocol (99-005) was approved by Rutgers Animal Care and Facilities Committee.

Plasma Corticosterone and Adrenocorticotropic Hormone Quantification

Plasma samples were used for costicosterone (CORT) and adrenocorticotropin hormone (ACTH) quantification during basal, restraint and post restraint time points. CORT plasma level was quantified using rodent Corticosterone ELISA (Immuno-Biological Labs, Cat# IB79175). ACTH plasma level was quantified using rodent ACTH ELISA (Phoenix Pharmaceuticals, Cat# EK 00121)

Immunofluorescence and Immunohistochemistry

Serial coronal sections of frozen brains were made using *Leica* cryostat at 20 μ m in thickness from stereotaxic plates 17 to plate 23 (Bregma -1.4 mm to -4.5 mm) spanning from the

paraventricular nucleus (PVN) to the arcuate nucleus (ARC) area. Three plate-equivalent sections were placed on each slide contained either one PF section, one AF section and one AF+M section or three sections of AD.

A total of 3 sections per brain were stained for all proteins detected within the PVN and 10 sections per brain were used for detection within the ARC. All primary antibodies were incubated overnight. Cytokine expression was detected by immunofluorescence using mouse polyclonal antibody for TNF- α (1:200, Abcam, Cat#Ab1793). Microglia proliferation was detected by double-immunofluorescence of BrdU and IBA1 using mouse monoclonal antibody for BrdU (1:200, BD Pharmingen; Cat# 555627) along with rabbit polyclonal antibody for IBA (1:400, Wako, Cat# 019-19741). AlexaFluor 594 donkey anti-mouse (1:500, Molecular Probes, Cat#A2120) and AlexaFluor 488 donkey anti-rabbit (1:1000, Molecular Probes, Cat#A20206) were used as secondary antibodies for immunoflorescence. After staining, the slides were mounted in DAPI (Vector Laboratories, Cat# H-1200) and covered with a 1mm coverslip.

Activated microglia were detected by immunohistochemistry using the mouse monoclonal CD11b (clone OX42) (1:50, Abd Serotec, Cat# MCA275). After overnight primary antibody incubation, sections were incubated with peroxidase-coupled anti-mouse Ig ImmPRESS reagent (Vector Laboratories, Cat# MP7402). Antigen localization was achieved by using the 3,3'-diaminobenzidine-peroxidase reaction and sections were then dehydrated and cover with a 1mm coverslip.

Pictures were taken using inverted microscope (Nikon EZ-C1 770, Gold Version). Expression intensity of TNF-alpha was quantified by Region of Interest (ROI) Statistics in a set area for each section to the left of the third ventricle in the paraventricular nucleus using the Nikon Imaging Software NIS-Elements AR version 4.11.00. Mean intensity was quantified and all groups were normalized to the control intensity (unstimulated Ad libitum). BRDU+IBA1, IBA1 and CD11b expressing cells were counted to the right of the third ventricle covering a total area of 1mm² for each section of the PVN and ARC region under 20X magnification.

Statistical Analysis

Graph Pad Prism version 4 software was used for statistical analysis of data. Quantitative Results are indicated as mean \pm SEM. The significance between treatment and controls was assessed using a two-way ANOVA between treatment and days of exposure. Post hoc analysis was performed using Bonferroni and Newman-Keuls post test. P < 0.05 was considered significant.

3.3 RESULTS

Acute neonatal ethanol exposure did not alter the stress response to acute restraint stress in adult males (Supplemental Figure 2). However, prolonged neonatal ethanol exposure significantly increased the response to acute restraint stress in adult males as quantified by the increase in CORT and ACTH plasma levels (Figure 14C &D). This effect was shown to be due to restraint as opposed to handling as demonstrated by the absence of hyper response following handling in alcohol exposed animals compared to controls (Figure 14A & B). Moreover, the peak in the response to acute restraint was shifted in AF animals suggesting delayed CORT inhibition (Supplemental Figure 2C). No changes were detected between groups in the absence of a stressor (basal levels) (Figure 14).



FIGURE 14. NEONATAL ETHANOL EXPOSURE ALTERS HPA RESPONSE TO ACUTE RESTRAINT STRESS IN ADULT MALES.

(A) CORT and (B) ACTH plasma levels in homecaged animals. (C) CORT and (D) ACTH plasma levels after 60 minutes of restraint stress. Data are mean \pm SEM of 5-6 independent observations. Two-way ANOVA followed by Bonferroni post-test revealed main effect of interaction between treatments and time for CORT; main effects of treatment, days of exposure and interaction for ACTH (P<0.0001). # P<0.05 and ### P<0.001 significantly different between AD and AF groups. b" P<0.001 significantly different between PF and AF groups.

We quantified the expression of pro-inflammatory cytokines in the PVN, the region of the hypothalamus where stress input initiates the activation of the HPA axis. Prolonged neonatal ethanol exposure significantly increased the expression of TNF- α in the PVN in response to acute restraint stress, as quantified by fluorescence intensity (Figure 15).



FIGURE 15. NEONATAL ETHANOL EXPOSURE INCREASES PRO-INFLAMMATORY CYTOKINE EXPRESSION IN THE PVN FOLLOWING ACUTE RESTRAINT STRESS IN ADULT MALES.

TNF-alpha expression was quantified by intensity of expression in the PVN after acute restraint stress. (A) Showing the representative immunofluorescence pictures of TNF-alpha expressing cells in PVN (20X). (B) Quantification of TNF-alpha intensity in PVN. All values were normalized against mean control values. Data are \pm SEM of 5 independent observations. One-way ANOVA followed by Bonferroni post-test revealed main effects of treatments and days of exposure (P<0.0001). ### P<0.001 significantly different between baseline and restraint. AF significantly different than AD and PF groups (* P< 0.05 and b P<0.05, respectively).

Neonatal ethanol exposure significantly increased the total microglia population in the PVN as quantified by the number of IBA1⁺ expressing cells (Figure 16). No difference in microglia proliferation during ethanol exposure was detected between AF animals and controls, as determine by the number of BrdU+IBA1 co-expressing cells in the PVN (Figure 17). A significant increase in CD11b⁺ expressing cells was detected in ethanol exposed animals at basal (Figure 18). Acute restraint stress slightly increased activated microglia in control groups; however, this effect was nearly doubled in alcohol exposed animals (Figure 18). No changes in

neither activated nor total microglia population were detected in the ARC between groups at basal levels; however, acute restraint stress significantly increased both activated and total microglia population in AF animals (Figure 19 & Figure 20). Acute restraint stress did not enhance microglial activation or total microglia in control animals (Figure 19 & Figure 20).



FIGURE 16. NEONATAL ETHANOL EXPOSURE INCREASES TOTAL MICROGLIA POPULATION IN THE PVN OF ADULT MALES.

Total microglia population was quantified by detection of $IBA1^+$ immnoreactive cells in the PVN. (A) Showing the representative immunofluorescence pictures (20X) of IBA1+ in the PVN of AD, PF, AF adult males. (B) Quantification of IBA1+ cell numbers in PVN. Data are \pm SEM of 5 independent observations. Two-way ANOVA followed by Bonferroni post-test revealed main effect of neonatal exposure (P=0.0003). * P< 0.05 significantly different between AD and AF groups. b P<0.05 and b' P<0.01 significantly different between PF and AF groups.





FIGURE 17. NEONATAL ETHANOL EXPOSURE DOES NOT INCREASE PROLIFERATION OF MICROGLIA IN THE PVN.

Microglial proliferation was quantified by detection of colocalization of IBA1+BrdU immnoreactive cells in the PVN. (A) Showing the representative immunofluorescence pictures (20X; 60X) of IBA1+BrdU in the PVN of AD, PF, AF adult males. (B) Quantification of IBA1+BrdU cell numbers in PVN. Data are \pm SEM of 5 independent observations.



FIGURE 18. NEONATAL ETHANOL EXPOSURE INCREASES MICROGLIAL ACTIVATION IN THE PVN OF ADULT MALES.

Activated microglia population was quantified by detection of CD11b⁺ immoreactive cells in the PVN. (**A**) Showing the representative immunofluorescence pictures (20X) of CD11b+ in the PVN of AD, PF, AF adult males. (**B**) Quantification of CD11b⁺ cell numbers in PVN. Data are \pm SEM of 5 independent observations. One-way ANOVA followed by Bonferroni post-test revealed main effects of treatment and neonatal exposure (P<0.0001). # P< 0.05 and ### P<0.001 significantly different between AD and AF. b" P<0.001 significantly different between PF and AF.



TOTAL MICROGLIA ARC



FIGURE 19. NEONATAL ETHANOL EXPOSURE INCREASES MICROGLIA POPULATION IN THE ARC IN RESPONSE TO ACUTE RESTRAINT STRESS IN ADULT MALES.

Total microglia population was quantified by detection of IBA1⁺ immnoreactive cells in the PVN. (A) Showing the representative immunofluorescence pictures (20X) of IBA1⁺ in the ARC of AD, PF, AF adult males. (B) Quantification of IBA1⁺ cell numbers in ARC. Data are \pm SEM of 5 independent observations. Two-way ANOVA followed by Bonferroni post-test revealed main effect of neonatal exposure (P=0.0079). # P<0.05 significantly different between baseline and restraint. * P< 0.05 significantly different between AD and AF groups.



FIGURE 20. NEONATAL ETHANOL EXPOSURE INCREASES MICROGLIAL ACTIVATION IN THE ARC OF ADULT MALES.

Activated microglia population was quantified by detection of CD11b⁺ immoreactive cells in the ARC. (A) Showing the representative immunofluorescence pictures (20X) of CD11b+ in the ARC of AD, PF, AF adult males. (B) Quantification of CD11b⁺ cell numbers in ARC. Data are \pm SEM of 5 independent observations. One-way ANOVA followed by Bonferroni post-test revealed main effect of neonatal exposure (P=0.0006). ### P<0.001 significantly different between baseline and restraint groups. ***P<0.001 significantly different between AD and AF groups. b" P<0.001 significantly different between PF and AF groups.

Lipopolysaccharide (LPS) is a component of the gram-negative bacteria well known for its ability to elicit an immune response in the periphery as well as the brain upon exposure (Buttini et al., 1996; Boyadjieva et al., 2009). LPS selectively binds to the CD14/MD2/TLR4 receptor complex to induce its inflammatory actions (Ospelt and Gay, 2009). Microglia, which are known to express TLR4 receptors, have been shown to be sensitive to LPS exposure and the binding of LPS on microglia induces pro-inflammatory signaling (Buttini et al., 1996). LPS exposure significantly increased the immune stress response in AF adult males compared to controls as quantified by the increase in CORT and ACTH plasma levels (Figure 21A & B). This alteration in immune stress response to LPS exposure was only present after prolonged neonatal ethanol exposure (Supplemental Figure 3). Minocycline pre-treatment during neonatal ethanol exposure prevented the hyper immune stress response observed in alcohol exposed animals (Figure 21A & B). Comparable to what we observed following acute restraint stress, LPS exposure significantly increased pro-inflammatory cytokine expression in the PVN of AF adult males compared to controls (Figure 22). This over-expression of pro-inflammatory cytokines in response to immune challenge was prevented by minocycline pre-treatment during neonatal ethanol ethanol exposure (Figure 22).

CORT 350 ###,* *b AD CORT PLASMA LEVEL (ng/mL) PF 250 AF ### C AD+M PF+M 150 AF+M 50 BASAL 2HRS LPS B. ACTH 175 AD ###.***.b' ACTH PLASMA LEVEL PF 125 ###,C AF (bg/mL) AD+M PF+M 75 AF+M 25 BASAL 2HRS LPS

A.

FIGURE 21. NEONATAL ETHANOL EXPOSURE ALTERS IMMUNE STRESS RESPONSE TO LPS IN ADULT MALES.

(A) CORT and (B) ACTH plasma levels after LPS challenge. Data are mean \pm SEM of 5-6 independent observations. Two-way ANOVA followed by Bonferroni post-test revealed main effects between treatments and neonatal exposure (P<0.0001). # P<0.05, ## P<0.01 and ### P<0.001 significantly different between baseline and LPS challenged. ***P<0.001 significantly different between AD and AF groups. b' P<0.01 and b'' P<0.001 significantly different between PF and AF groups. b P<0.05 significantly different between PF and PF+M groups. c P<0.05 and c'' P<0.001 significantly different between AF and AF+M groups.



FIGURE 22. NEONATAL ETHANOL EXPOSURE INCREASES PRO-INFLAMMATORY CYTOKINE EXPRESSION IN THE PVN FOLLOWING LPS IN ADULT MALES.

TNF-alpha expression was quantified by intensity of expression in the PVN after LPS challenge. (A) Showing the representative immunofluorescence pictures of TNF-alpha expressing cells in PVN (20X). (B) Quantification of TNF-alpha intensity in PVN. All values were normalized against mean control values. Data are \pm SEM of 5 independent observations. One-way ANOVA followed by Bonferroni post-test revealed main effects of treatments and days of exposure (P<0.0001). ## P< 0.01 significantly different between baseline and LPS exposed. AF significantly different compared to AD, PF and AF+M (* P<0.05, b P<0.05 and c P<0.05, respectively).

LPS slightly increased total microglia population in the ARC similarly in AD and AF animals (Figure 23). Although alcohol exposure increases total microglia population in the PVN at basal, LPS did not enhance the number of microglia population in the PVN upon exposure (Figure 23). Minocycline pre-treatment normalized the effects of ethanol on the total number of microglia in the PVN in neonatally exposed animals (Figure 23). LPS exposure significantly increased the number of activated microglia in the PVN in all groups; however, the number of activated microglia in the PVN was significantly higher in AF animals compared to controls (Figure 24). In contrast, LPS exposure only increased the number of CD11b⁺ expressing cells in

the ARC of AF animals (Figure 25); this increase in activated microglia in the ARC was normalized to values comparable to controls by minocycline pre-treatment during ethanol exposure (Figure 25).



FIGURE 23. MINOCYCLINE PRE-TREATMENT PREVENTS NEONATAL ETHANOL INDUCED INCREASE OF TOTAL MICROGLIA POPULATION IN THE PVN OF ADULT MALES.

Total microglia population was quantified by detection of $IBA1^+$ immnoreactive cells in the PVN and ARC. (A) Showing the representative immunofluorescence pictures (20X) of $IBA1^+$ in the PVN and ARC of AD, PF, AF and AF+M adult males. (B) Quantification of IBA1+ cell numbers in PVN. (C) Quantification of IBA1+ cell numbers in ARC. Data are \pm SEM of 5 independent observations. Two-way ANOVA followed by Bonferroni post-test revealed main effect of neonatal exposure for PVN (P=0.0001); main effect of treatment for ARC (P<0.0001). # P< 0.05 significantly different between baseline and LPS exposure. **P<0.01 significantly different between PF and AF groups. c' P<0.01 significantly different between AF and AF+M groups.

B.



FIGURE 24. NEONATAL ETHANOL EXPOSURE INCREASES MICROGLIAL ACTIVATION IN THE PVN OF ADULT MALES.

2HRS LPS

BASAL

Activated microglia population was quantified by detection of CD11b⁺ immoreactive cells in the PVN. (A) Showing the representative immunofluorescence pictures (20X) of CD11b+ in the PVN of AD, PF, AF and AF+M adult males. (B) Quantification of CD11b⁺ cell numbers in PVN. Data are \pm SEM of 5 independent observations. One-way ANOVA followed by Bonferroni post-test revealed main effects of treatment and neonatal exposure (P<0.0001). ### P<0.001 significantly different between baseline and LPS exposure. * P< 0.05 and **P<0.01 significantly different between AD and AF groups.



FIGURE 25. NEONATAL ETHANOL EXPOSURE INCREASES MICROGLIA ACTIVATION IN THE ARC OF ADULT MALES.

Activated microglia population was quantified by detection of CD11b⁺ immoreactive cells in the ARC. (A) Showing the representative immunofluorescence pictures (20X) of CD11b+ in the ARC of AD, PF, AF and AF+M adult males. (B) Quantification of CD11b⁺ cell numbers in ARC. Data are \pm SEM of 5 independent observations. Two-way ANOVA followed by Bonferroni post-test revealed main effects of treatment and neonatal exposure (P=0.0005). ### P<0.001 significantly different between baseline and LPS exposure. AF significantly compared to AD, PF and AF+M groups (***P<0.001, b" P<0001 and c" P<0.001, respectively).

3.4 DISCUSSION

We demonstrate for the first time that ethanol exposure during the third trimester equivalent results in hyper response to acute restraint stress in adult males. In addition, we confirmed that ethanol exposure during the third trimester equivalent results in the hyper response to acute immune challenges, as demonstrated by the increased CORT and ACTH plasma levels in response to LPS in AF animals. Interestingly, neonatal ethanol exposure does not result differences in basal CORT or ACTH; the observed differences were only detected in the presence of a stimulus. This alteration in HPA axis response to acute stress was dependent on the length of neonatal ethanol exposure. In our postnatal model, single exposure to alcohol in neonates did not alter the HPA response to neither acute restraint stress nor acute LPS challenge in adulthood. However prolonged ethanol exposure during the neonatal period increased sensitivity to acute stressors in adulthood, and resulted in significant changes in HPA axis response following five days of neonatal ethanol exposure. These findings suggest that prolonged exposure to ethanol is necessary to reprogram the HPA axis in neonates.

Moreover, we provide evidence for the first time that neonatal ethanol exposure may reprogram microglia sensitivity to subsequent stimuli. Prolonged neonatal ethanol exposure increased pro-inflammatory cytokine expression, specifically TNF- α , in the PVN in response to both acute restraint and LPS challenge. While there were no basal differences between groups, prolonged neonatal ethanol exposure increased the number of activated microglia in the ARC in response to acute restraint and LPS challenge. Acute restraint stress increased the total number of microglia in the ARC of AF adult males; however, LPS challenge increased total microglia in the ARC similarly in all groups. Interestingly, ethanol exposed animals showed basal increase in the number of activated microglia in the PVN compared to control animals; acute restraint stress and LPS challenged augmented the number of activated microglia in the PVN. These results suggest that ethanol induced changes in microglia sensitivity or response to subsequent stimuli vary depending on the region of the CNS as well as the stimulus.

In contrast to the ARC, prolonged neonatal ethanol exposure increased basal total microglia population in the PVN of adult male rats. Neither acute restraint stress nor LPS challenge increase total microglia cell number in the PVN of any groups. Although the increase in total microglia in the PVN does not appear to be due to increased proliferation during ethanol exposure, microglia proliferation may have taken place after ethanol exposure. *In vitro* studies have consistently shown that microglial activation can induce proliferation (Gomez-Nicola et al., 2013), however emerging evidence illustrates that ethanol induced microglia proliferation may take place during abstinence rather than during exposure (Zhao et al., 2013; McClain et al., 2011; Crews and Nixon, 2009).

Most importantly, we demonstrated for the first time that minocycline pre-treatment during neonatal ethanol exposure prevents alterations in HPA axis as well as increases in microglial activation in response to stressors in adulthood. Minocycline pre-treatment prevented the increased CORT and ACTH response to LPS challenge. Additionally, minocycline pretreatment prevented the increase of total microglia in the PVN of adult males as well as the increase in microglial activation in response to LPS challenge in both the PVN and ARC. These findings support our hypothesis that initial activation of microglia during neonatal ethanol exposure plays a role in the alterations in HPA axis response to stimuli in adulthood.

HPA activation induces and/or enhances the activation of local neuronal and immune cells, including microglia in the hypothalamus (Chang et al., 2009). It is well accepted that microglia initiate and propagate inflammatory signaling that maintain HPA activation and elicit behavioral changes following immune or psychological stressors (Chang et al., 2009; Song and Wang, 2011). In addition to downregulating HPA activation by suppressing CRH release in the PVN and ACTH release from the pituitary, glucocorticoids (CORT in rodents) also play a role in modulating inflammatory signaling by suppressing immune activation. Recent evidence shows that CORT binds to microglia and suppresses their activation, thereby reducing the inflammatory signaling (Nakatani et al., 2012; Sugama et al., 2012). Moreover, Sugama and collegues (2012)

previously demonstrated that CORT limits microglial activation following acute restraint stress (Sugama et al., 2012). In this study, restraint stress induced minimal activation of microglia in the hypothalamus, as quantified by CD11b⁺ expressing cells. However, when animals were adrenalectomized to prevent CORT release upon HPA activation, the number of CD11b⁺ expressing cells nearly tripled following acute restraint stress (Sugama et al., 2012). Interestingly, we observed increased microglial activation following acute restraint in AF animals, even in the presence of high CORT plasma levels suggesting delayed inhibition. Taken together, these findings provide evidence that ethanol induced disruptions in HPA axis may partially be due to altered microglia sensitivity.

Neonatal ethanol exposure may permanently alter microglia sensitivity to subsequent stimuli by reducing the effect of inhibitory factors such as CORT. Neonatal exposure to various stimuli has been well documented to cause lifelong alterations in HPA axis response. Permanent changes in glucocorticoid receptors expression have been implicated in many developmental effects of neonatal stress (Seckl and Meaney, 2004). Numerous reports of cell and tissue specific changes in GR expression support the hypothesis that alterations in GR expression may play a role in abnormal HPA response following neonatal exposure to stressors and toxins (Xiang and Zhang, 2013). CORT has been shown to primarily regulate microglial activation and proliferation by binding to GR as opposed to its alternative receptor, MR (Blandino et al., 2009; Nakatani et al., 2012). Therefore, microglia sensitivity following neonatal ethanol exposure may partially be due to permanent alterations in GR expression.

Initial activation of microglia has been shown to increase microglia responses to subsequent exposure to LPS in cultures (Parajuli et al., 2012), where CORT elevation is not present following challenge. Therefore, alterations in GR receptor expression following neonatal stress may not be the primary factor in microglia mediated alterations in HPA axis. Hence, the alteration in HPA response following neonatal ethanol exposure could result from increases in receptor of stimulatory factors (ex. LPS) leading to the enhancement of inflammatory signaling

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upon stress induced activation in adulthood. This mechanism will be further discussed in Chapter 5.

3.5 CHAPTER 3 SUPPLEMENTAL DATA



SUPPLEMENTAL FIGURE 2. ACUTE NEONATAL ETHANOL EXPOSURE DOES NOT ALTER HPA RESPONSE TO ACUTE RESTRAINT STRESS IN ADULT MALES.

CORT response curve to acute restraint stress. CORT plasma levels after acute restraint stress in adult males after (A) 1 Day, (B) 3 Days and (C) 5 Days of neonatal ethanol exposure. Basal (B), Restraint stress timepoints (15R, 30R and 60R) and Post restraint timepoints (30P, 60P, 120P). Data are mean \pm SEM of 5-6 independent observations. Two-way ANOVA followed by Bonferroni post-test revealed main effect between timepoints for 1 Day and 3 Days (P<0.001 and P<0.0485, respectively); main effects of neonatal exposure and timepoints for 5 Days (P<0.0001). *P<0.05 and **P<0.01 significantly different between groups.



SUPPLEMENTAL FIGURE 3. ACUTE NEONATAL ETHANOL EXPOSURE DOES NOT ALTER IMMUNE STRESS RESPONSE TO LPS IN ADULT MALES.

CORT plasma levels after LPS challenge. Data are mean \pm SEM of 6 independent observations. Two-way ANOVA followed by Bonferroni post-test revealed main effects between treatments (P<0.0001). ### P<0.001 significantly difference between baseline and LPS exposure.
CHAPTER 4

4.1 INTRODUCTION

Alcohol is a well known neurotoxin with extensively documented harmful effects among children exposed during development, including neuro-cognitive deficits, neuronal injury and neurodegeneration (Weinberg et al, 2009; Sarkar et al, 2008). Although the effects of ethanol exposure are well acknowledged, the specific mechanisms behind its neurotoxic effects are not well understood. We have previously shown that ethanol exposure during the third trimester equivalent induces apoptosis of developing β -endorphin (BEP) neurons in the ARC of the hypothalamus (Sarkar et al, 2008). Proopiomelanocortin (POMC) neurons, which secrete the endogenous opioid BEP in the hypothalamus, control a variety of functions including energy metabolism, stress control and immune functions. It has been shown that prenatal and postnatal ethanol exposed animals are unable to appropriately respond to stress as adults, most likely due to alterations in the function of the HPA axis (Zhang et al., 2005; Sarkar et al., 2008). The reduction of BEP producing neurons may contribute to ethanol induced HPA dysfunction (Sarkar et al., 2008).

Although the role of ethanol induced microglial activation in the developing brain is not fully understood, ethanol induced microglial activation has been suggested to contribute to neurodegeneration and neurotoxicity. Pure microglia cultures have been shown to produce and release neurotoxic factors that enhance neuronal damage and apoptotic signaling in neurons in response to ethanol exposure (Boyadjieva., 2010; 2012; Pascual et al., 2011, Fernandez-Lizarbe, 2013). Using conditioned media of ethanol exposed microglia culture, we have previously demonstrated that ethanol increase microglia production of pro-inflammatory cytokines and reactive oxygen species, and these factors enhance ethanol induced neurotoxicity in developing hypothalamic neurons (Boyadjieva and Sarkar., 2010, 2013a, 2013b). The interactions between opioid and microglia, which express opioid receptors, have also been investigated. The binding of opioids to microglia have been shown to regulate their activity (Mika, 2008; Vranken, 2012; Cooper et al., 2012). Opioids can suppress or enhance microglial activation, resulting in either neurotrophic or neurotoxic effects depending on the type of opioid ligand as well as the dose and length of exposure (Hutchinson et al., 2011). We have recently found that the activation of mu opioid receptor (MOR) augment the neurotoxic effects of ethanol induced microglial activation through the enhancement of pro-inflammatory cytokine actions (Boyadjieva and Sarkar, unpublished). Interestingly, delta opioid receptor (DOR) activation suppresses the neurotoxic effects of ethanol induced microglia (Boyadjieva and Sarkar, unpublished). This data reaffirms the complexity of opioid interaction with microglia and suggests that ethanol induced microglia neurotoxicity may be mediated by opioids.

BEP, which binds to both MOR and DOR, is known to be released in response to stress. Its primary function during stress response is to downregulate the activation of the HPA axis by suppressing CRH release from CRH-producing neurons in the PVN (Barfield et al., 2010; 2013). BEP has also been shown to regulate the activation of the peripheral innate immune system (Sarkar et al., 2011). Although correlative data suggest a relationship between the suppression of inflammatory signaling and the elevation of BEP (Sarkar et al., 2011; Labuzek et al., 2013), the possible role of BEP in the regulation in the CNS innate immune system has not been fully investigated. Here, we determined whether ethanol induced apoptosis of β -endorphin expressing neurons contribute to the hyper response to stress observed in AF adult males. In addition, we sought out to determine whether the reduction of β -endorphin expressing neurons contribute to the increased activation of microglia in response to acute stress.

4.2 MATERIALS AND METHODS

Animal Use

Postnatal Alcohol Exposure Model

Sprague-Dawley females and males were purchased from Charles River and mated in house. The rats were housed in 12h light/ 12h dark cycles (lights on 7:00am and off 7:00 pm) on a constant temperature (22°C) throughout the study. One day after birth, male neonates were divided into one of four groups; Ad libitum (AD), Pair-fed (PF), Alcohol-fed (AF) and Alcohol-Fed + Minocycline (AF+M). The AD group were only nursed by the mother. The PF, AF and AF+M neonates were fed twice daily by intubation with a milk formula (PF) or with milk formula containing alcohol (11.34% v/v) (AF and AF+M) in addition to nursing for up to five days with two hours between feedings. The AF+M group received a minocycline pre-treatment one hour prior to the first feeding on each day by subcutaneous injection of minocycline solution (45 μ g/kg bodyweight). Each brain was collected one hour after last feeding and immediately frozen and stored at -80°C.

Differentiation of β -endorphin cells from neuronal stem cells

Hypothalamic neural stem cells were differentiated β -endorphin neurons in culture as previously described (Sarkar et al., 2008). Neuronal stem cells were isolated from hypothalamic tissue of E17 fetal rat brains. Cyclic adenosine monophosphate (cAMP) and pituitary adenylate cyclase-activating polypeptide (PACAP) were used to differentiate neuronal cells into β endorphin neurons. Differentiated cortical cells from cortical tissue of E17 fetal rat brains were used as a control for transplantation. Prior to transplantation, differentiated BEP cells were dissociated and resuspended at a concentration of 20,000 viable cells/µL in HEPES-buffered Dulbecco's modified Eagle's medium (DMEM)-containing serum supplement (30 nmol/L selenium, 20 nmol/L progesterone, 1 µmol/L iron-free human transferrin, 5 µmol/L insulin, 100 μmol/L putrescine, and antibiotics), cAMP (10 μmol/L), and PACAP (10 μmol/L) for the transplantation. Cells were placed on ice throughout the grafting session. Cell viability, assessed by the trypan blue exclusion assay, was routinely more than 90%. The composition of the differentiated cultures, with respect to the absence of undifferentiated neural stem cells and the presence of mature BEP-producing cells, was verified before grafting by staining for the immature neural marker nestin and/or vimentin, and for BEP using immunocytochemistry as described previously (Sarkar et al., 2008).

Transplantation of Differentiated Stem Cells

Neonates were treated with ethanol as described above. The number of neonates was normalized to six pups per dams and each litter contained different treatment groups to reduce litter effects. After 5 days of ethanol treatment, male neonates were kept with dam until PD 21 and were then weaned into housed in groups of two or three animals per cages. At PD 30, animals were anesthetized with sodium pentobarbital (50-70 mg/kg, i.p., Henry Schein) and injected with 1.0 μ L of cell suspension (20, 000 cells per lobe) into PVN lobes (the coordinates were set 0.5 mm from the midline, 1.8 mm behind the bregma, 0.5 mm lateral of the bregma, and 7.5 mm below the cortex) using a 5 μ L Hamilton syringe. The dura was closed with 9–0 suture, the muscle was reapposed, and the skin was closed with wound clips. Animal surgery and care were performed in accordance with institutional guidelines and complied with National Institutes of Health policy.

Acute Restraint Stress

At PD 90, adult males were subjected to 60 minutes of restraint stress followed by a recovery period where the animals were returned into their homecages. Animals were habituated with homecages for three days before stress experiments. Tail blood was collected at 0 (Basal), 15, 30 and 60 min of restraint followed by 30, 60 and 120min after restraint in EDTA containing

tubes. Each brain was collected after 120 min post restraint and immediately frozen. Plasma samples and brain were stored at -80°C.

Plasma Corticosterone and Adrenocorticotropic Hormone Quantification

Plasma samples were used for CORT and ACTH quantification during basal, restraint and post restraint time points. CORT plasma level was quantified using rodent Corticosterone ELISA (Immuno-Biological Labs, Cat# IB79175). ACTH plasma level was quantified using rodent ACTH ELISA (Phoenix Pharmaceuticals, Cat# EK 00121)

Immunofluorescence

POMC neurons were detected by immunofluorescence using goat polyclonal antibody (1:400, Santa Cruz, Cat# SC-18262) and β -endorphin expressing neurons were detected using rabbit polyclonal antibody (1:1000, Peninsula Laboratories, Cat#T-4045).

Pictures were taken using inverted microscope (Nikon EZ-C1 build 770, Gold Version). Total number of POMC and BEP expressing cells were counted in a total area of 1mm² per section in the ARC region.

Immunohistochemistry

Serial coronal sections frozen brains were made using *Leica* cryostat at 20 μ m in thickness from stereotaxic plates 17 to plate 23 (Bregma -1.4 mm to -4.5 mm) spanning from the paraventricular nucleus (PVN) to the arcuate nucleus (ARC) area.

Sections from brains of transplanted animals were collected and two plate-equivalent sections were placed on each slide containing one Ad libitum transplanted with cortical cells (AD-CC) or β -endorphin cells (AD-BEP) section, one Pair fed or Alcohol fed transplanted with cortical cells (PF-CC and AF-CC, respectively) or one Pair fed or Alcohol fed transplanted with β -endorphin cells (PF-BEP and AF-BEP, respectively) section.

Activated microglia were detected by immunohistochemistry using the mouse monoclonal CD11b (clone OX42) (1:50, Abd Serotec, Cat# MCA275). After overnight primary antibody incubation, sections were incubated with peroxidase-coupled anti-mouse Ig ImmPRESS reagent (Vector Laboratories, Cat# MP7402). Antigen localization was achieved by using the 3,3'-diaminobenzidine-peroxidase reaction and sections were then dehydrated and cover with a 1mm coverslip. Average number of CD11b expressing cells were counted in a total area of 1mm² per section to the right of the third ventricle in the PVN and ARC region.

Statistical Analysis

Graph Pad Prism version 4 software was used for statistical analysis of data. Quantitative Results are indicated as mean \pm SEM. The significance between treatment and controls was assessed using a two-way ANOVA with post hoc analysis using Bonferroni and Newman-Keuls post test. P < 0.05 was considered significant.

4.3 RESULTS

BEP is produced by POMC neurons in the ARC of the hypothalamus. We have previously shown that neonatal ethanol exposure reduces POMC gene expression in rodents (Agapito et al., 2013). As expected, neonatal ethanol exposure resulted in a reduction in POMC expressing neurons and a trend towards a decrease in BEP expressing neurons in the ARC of neonates (Figure 26 and Figure 27B, respectively). This ethanol induced reduction of BEP expressing neurons was permanent, as determined by the decrease in BEP expressing neurons in the ARC of adult males (Figure 27C).





FIGURE 26. ETHANOL EXPOSURE DECREASES THE NUMBER OF POMC EXPRESSING NEURONS IN THE ARC OF NEONATES.

POMC cell number was quantified by the detection of POMC expressing neurons. (A) Showing the representative immunofluorescence pictures of POMC expressing cells in ARC (10X). (B) Quantification of POMC expressing neurons in ARC. Data are \pm SEM of 5 independent observations. Two-way ANOVA followed by Bonferroni post-test revealed main effects of days of exposure and interactions between treatments and days of exposure (P<0.0001). *** P<0.001 significantly different between AD and AF groups. b' P<0.01 and b'' P<0.001 significantly different between PF and AF groups.



FIGURE 27. ETHANOL EXPOSURE PERMANENTLY DECREASES THE NUMBER OF BEP EXPRESSING NEURONS IN THE ARC.

BEP cell number was quantified by the detection of BEP expressing neurons. (A) Showing the representative immunofluorescence pictures of BEP expressing cells in ARC (10X). (B) Quantification of BEP expressing neurons in ARC in neonates. (C) Quantification of BEP expressing neurons in ARC in adult males. Data are \pm SEM of 5-6 independent observations. One-way ANOVA followed by Bonferroni post-test revealed main effects of days of exposure and interactions between treatments and days of exposure (P=0.0356). * P<0.05 significantly different between AF and AF+M groups.

We have previously provided evidence that transplantation of differentiated BEP expressing neurons into the PVN of adult males prevents the hyper stress response observed after prenatal ethanol exposure (Boyadjieva et al., 2009). As previously demonstrated, prolonged neonatal ethanol exposure increased the response to acute restraint stress in adult males, as quantified by the increased levels in CORT and ACTH plasma levels (Figure 28A & B). Males transplanted with differentiated cortical neurons (AF-CC) remained hyper responsive to acute

A.

restraint stress (Figure 28). Moreover, AF-CC animals also demonstrated a shift of peak in response to acute restraint stress suggesting delayed inhibition (Supplemental Figure 4A).

In contrast, adult males transplanted with differentiated BEP neurons (AF-BEP) showed decrease responses to acute restraint stress, as quantified by CORT and ACTH plasma levels (Figure 28A& B). In addition, transplantation of BEP neurons reestablished the proper curve response to acute restraint stress in AF-BEP animals, although overall stress response was reduced in these animals (Supplemental Figure 4B).



FIGURE 28. BEP TRANSPLANTATION PREVENTS HYPER RESPONSE TO ACUTE RESTRAINT STRESS IN NEONATAL ETHANOL EXPOSED ADULT MALES.

(A) CORT and (B) ACTH plasma levels in homecaged animals. (C) CORT and (D) ACTH plasma levels after 60 minutes of restraint stress. Data are mean \pm SEM of 5-6 independent observations. Two-way ANOVA followed by Bonferroni post-test revealed main effect of interaction between treatments and time for CORT; main effects of treatment, days of exposure and interaction for ACTH (P<0.0001). ## P<0.01 and ### P<0.001 significantly different between baseline and restraint. **P<0.01 and ***P<0.001 significantly different compared to AD groups. a P<0.05 and a' P<0.01 significantly different compared to PF group. d P<0.05 and d'' P<0.001 significantly different between AF-BEP and AF-BEP and AF-CC groups.

4.4 WORKING HYPOTHESIS AND DISCUSSION

We provide evidence that ethanol exposure during the neonatal period results in fewer POMC and BEP expressing neurons in the ARC of neonates. The number in BEP neurons remains reduced in AF animals well into adulthood. Moreover, we demonstrate that minocycline pre-treatment prevents the reduction of BEP expressing neurons in adult males. This data supports our previous findings that ethanol exposure during the third trimester equivalent results in permanent reduction of BEP neurons by increasing apoptotic signaling mediated by microglial activation in the ARC (Sarkar et al., 2008; Boyadjieva and Sarkar, 2010).

We demonstrate for the first time that BEP transplantation reduces the hyper response to stressors caused by neonatal ethanol exposure. This data supports our previous findings that BEP transplantation can ameliorate HPA axis responses to either immune challenge (Boyadjieva et al., 2009) or psychological stressors (Logan and Sarkar, unpublished) in prenatal ethanol exposed animals. Furthermore, this data supports our hypothesis that BEP apoptosis during neonatal ethanol exposure contributes to the hyper response to stressors during adulthood.

It is well established that activation of the HPA axis initiates the release of CRH from CRH producing neurons as well as BEP from POMC producing cells including the POMC neurons of the hypothalamus (Barfield et al., 2013). CRH release initiates a signaling cascade that results in CORT release from the adrenal cortex. The activation of the HPA axis is partially maintained by local and global cytokine production.

Psychological and physiological stress is often accompanied by immune activation. Immune activation in the periphery stimulates pro-inflammatory cytokine release, which can either enter the CNS by volume diffusion, cytokine transporters at the blood brain barrier (BBB) or binding to receptors at the BBB to initiate inflammatory signaling in the CNS (Kasten-Jolly and Lawrence, 2011). Inflammatory signaling can also be initiated in the CNS by activation of neuronal and CNS immune cells, including microglia (Kwon et al., 2007; Sugama et al., 2009; 2012). CRH binds to microglia in the CNS and induces their activation. Stress induced microglial activation has been shown to increase pro-inflammatory cytokines and propagate the HPA axis signaling (Sugama et al., 2009; 2012). Our data suggest that increased proinflammatory cytokine production in the PVN may contribute to hyper response to both psychological and physiological stressors in AF animals. This increase in pro-inflammatory signaling may be due to increased or dysregulated microglial activation. This hypothesis is supported by evidence of increased microglial activation in the hypothalamus following acute restraint stress and LPS exposure in our postnatal ethanol model.

CRH release is downregulated in many ways including diffusion of plasma CORT into the CNS, activation of GABAergic neurons in the PVN as well as BEP release in the PVN (Oswald and Wand, 2004). BEP stress regulation may be consisting of two roles. First, it is known that BEP producing neurons in the ARC release the BEP into the PVN, where BEP binds to opioid receptors on CRH neurons and modulate the release of CRH (Tsagarokis et al., 1990). Second, BEP-expressing neurons could mediate the local production of pro-inflammatory cytokines in the hypothalamus by directly modulating microglial activation (Representative model; Figure 29). The modulation of microglial activation could be regulated through the differential cleavage of POMC peptide known to regulate inflammatory signaling. Such peptides include α -MSH, which is released upon stress activation and has been shown inhibit NFkB signaling and in turn suppress inflammatory signaling (Kasten-Jolly and Lawrence, 2011). Alternatively, local BEP release may increase binding to microglia and suppress their activation. If the latter is true, stress may also induce differential expression of opioid receptors on microglia.



FIGURE 29. MODEL OF B-ENDORPHIN REGULATION OF HPA ACTIVATION.

Mechanism by which B-endorphin inhibits HPA activation (A) B-endorphin inhibits the release of CRH from CRH producing in the PVN. (B) POMC peptides including B-endorphin may regulate the production of pro-inflammatory cytokines in response to stress by suppressing the activation of microglia in the PVN

MOR agonists, such as morphine, have been shown to increase microglial activation and increase the expression of MOR (Matsushita et al., 2013). As discussed before, we have recently found that MOR and DOR agonist result in differential microglial activation. Activation of microglia is known to induce alterations in surface protein and receptor expression (Cribbs et al., 2012; Fernandez-lizarbe et al., 2013; Hutchinson et al., 2011; Badoer 2011). Stress induced microglial activation may increase DOR expression, leading to suppression of pro-inflammatory signaling in microglia upon binding of BEP. Therefore, ethanol induced reduction of BEP cells may result in decrease regulation of microglial activation following stress.

We have begun to investigate the interaction of BEP and microglia following acute stress. Preliminary data suggests that BEP transplantation reduces restraint stress-induced microglial activation in AF-BEP animals compared to AF (data not shown). More extensive analysis needs to be completed in order to determine whether BEP suppression of HPA axis is partially due to BEP suppression of microglia pro-inflammatory signaling.

4.5 CHAPTER 4 SUPPLEMENTAL DATA

A.

B.



SUPPLEMENTAL FIGURE 4. EFFECTS OF CELL TRANSPLANTATION ON HPA STRESS RESPONSE TO ACUTE RESTRAINT STRESS.

CORT response curve to acute restraint stress. CORT plasma levels after acute restraint stress in adult males after (A) Cortical Cell (CC) transplantation and (B) β -endorphin (BEP) transplantation. Basal (B), Restraint stress timepoints (15R, 30R and 60R) and Post restraint timepoints (30P, 60P, 120P). Data are mean \pm SEM of 5 independent observations. Two-way ANOVA followed by Bonferroni post-test revealed main effects of neonatal exposure and timepoints for 5 Days (P<0.0001); main effect between timepoints BEP transplanted animals (P=0.0003). **P<0.01 and ***P<0.001 significantly different between groups.

5.1 ETHANOL EFFECTS OF MICROGLIAL ACTIVATION ON NEONATES

The concept that exposure to environmental factors during development can result in lifelong outcomes in exposed individuals is well accepted. Environmental risk factors known to have detrimental effects on the developing fetus include but are not limited to nutrition, exposure to tobacco and substances of abuse such as alcohol. Exposure to alcohol during developmental period results in alcohol effects collectively defined as fetal alcohol spectrum disorder (FASD) (Ornoy and Yacobi, 2011). Ethanol exposure during development result in severe neurobehavioral defects in the fetus outweigh those of cocaine, heroin and marijuana (nofas.org, 2013).

The detrimental effects of ethanol exposure during development include neuronal damage and neurodegeneration (Weinberg et al., 2009; Sarkar et al., 2008). We have previously shown that prenatal and postnatal ethanol exposure results in reduction of POMC gene expression and BEP expressing neurons in the ARC of the hypothalamus in rodents (Sarkar et al., 2008; Govorko et al., 2012; Bekdash et al., 2013; Agapito et al., 2013). The reduction in BEP expressing cell number in the ARC is thought to be partially due to increased ethanol induced apoptotic signaling in the hypothalamus of neonates. Here, we provide evidence to support our previous findings showing that BEP expressing neuronal cell number are permanently reduced in alcohol exposed animals due to a reduction of POMC neurons in the hypothalamus.

Microglial activation has been implicated in many neurodegenerative diseases due to their productions of various factors such as nitric oxide NO, ROS, cytotoxic and/or proinflammatory factors (Cribbs et al., 2012; Beynon and Walker, 2012; Giunta et al., 2012). We have previously demonstrated that *in vitro* ethanol exposure induces microglial production and release of ROS, NO and inflammatory factors such as MIP1 α , MIP2, IL6 and TNF- α (Boyadjieva and Sarkar, 2008). Consistent with these *in vitro* data, we demonstrate that ethanol exposure during the neonatal period (human third trimester equivalent), results in microglial activation. We report for the first time that ethanol exposure alternatively activate microglia depending on the length of exposure, resulting in differential cytokine production and release in the mediobasal hypothalamus (Representative model, Figure 30).



FIGURE 30. MODEL OF ETHANOL INDUCED MICROGLIAL ACTIVATION IN THE MEDIOBASAL HYPOTHALAMUS OF NEONATES.

Ethanol activates immature microglia and induces the production of cytokines in the hypothalamus of neonates. Cytokine profile is altered depending of the length of exposure. Abbreviations: EtOH, Ethanol; MCP1, Monocyte Chemotactic Protein 1; IL6, Interleukin 6; TNF- α , Tumor Necrosis Factor-alpha; IL10, Interleukin 10.

Previous analyses of the effects of ethanol induced activation of microglia cells revealed that their activation may have a critical role in ethanol induced neurotoxicity of developing hypothalamic neurons (Boyadjieva and Sarkar, 2010). Ethanol induced apoptosis of hypothalamic neurons was enhanced in the presence of conditioned media from ethanol treated microglia. The potentiation of microglia conditioned media was prevented by immunoneutralization of TNF- α production and release in microglia, demonstrating that TNF- α plays a critical role in ethanol induced microglia neurotoxicity (Boyadjieva and Sarkar, 2010). In addition, we previously reported that ethanol induced apoptotic signaling increased after two days

and peaked by four days of exposure during neonatal period (Sarkar et al., 2008). Interestingly, we discovered that TNF-alpha elevation in AF neonates correlated with our previously described period of ethanol induced apoptosis of hypothalamic neurons. Taken together, our data support other reports of "window of vulnerability" to alcohol apoptotic effects in neonates (Goddlett and Eilers, 1997; Clancy et al., 2001; Kane et al., 2011). Microglial involvement in ethanol induced neurodegeneration of hypothalamic neurons still needs to be confirmed *in vivo*. This hypothesis will be addressed by suppressing apoptotic signaling through TNF- α neutralization in alcohol fed neonates.

Dysregulated and/or persistent microglial inflammatory signaling have been associated with various diseases (Cribbs et al., 2012; Beynon and Walker, 2012; Giunta et al., 2012). The inflammatory signaling is regulated in the CNS through the production and release of several factors including inhibitors of cytokine synthesis, cytokine antagonists and anti-inflammatory cytokines (Chang et al., 2009). Amongst these, the anti-inflammatory cytokine IL10 has been most extensively studied (Chang et al., 2009). It is well established that IL10 inhibits proinflammatory cytokine production through the inhibition of MAPK and NFkB signaling in microglia (Heyen et al., 2000). Recently, increasing evidence shows that IL10 induces alternative activation of microglia which resembles the M2 activated phenotype of peripheral macrophages (Boche et al., 2013; Bilbo, 2013; Harry, 2013; Marshall et al., 2013). Not surprisingly, the increase in IL10 observed in AF neonates correlated with the reduction of pro-inflammatory cytokine expression. We detected a significant increase in IL10 after five days of ethanol exposure which correlated with an increase CD11b⁺ expressing cells in AF neonates. This increase number of CD11b⁺ expressing cells may be representative of alternatively activated microglia mainly responsible for the attenuation of ethanol induced inflammatory signaling and the removal of apoptotic bodies accumulated from apoptotic neuronal cell death in previous days of ethanol exposure. This hypothesis may be address by quantifying the expression of surface

receptors known to be upregulated in microglia during phagocytosis of apoptotic cells (Figure 31) (Sierra et al., 2013).



FIGURE 31. RECEPTORS INVOLVED IN MICROGLIAL PHAGOOCYTOSIS OF APOPTOTIC CELLS.

Receptors have been classified into major functional/structural groups. Phosphatidylserine (PS) receptors, integrin receptors, Ig superfamily receptors and Scavenger and related receptors. Adaptation of Figure 2 from Sierra et al., 2013.

Lastly, the alterations of microglia modulators may contribute to altered microglial activation and/or responses. Various modulators are known to suppress microglial inflammatory signaling in the adult brain (Lyons et al., 2007; Bachsetter et al., 2011; Wong, 2013). However, only a subset of the receptors necessary for signaling are present on the immature microglia (Ransohoff, 2011; Paolicelli et al., 2011; Mizutani et al., 2012; Shrivastava et al., 2012). Amongst these, CD200-CD200R (Shrivastava et al., 2012) and CX3CL1-CX3CR1 (Ransohoff, 2011) signaling play critical roles in normal development as well as anti-inflammatory signaling in the developing brain. The increased activation of both CD200-CD200R and CX3CL1-CX3CR1 signaling have been shown to regulate microglial inflammatory signaling throughout life (Hoarau et al., 2011; Jaerve and Müller, 2012; Wong, 2013). Upon stressful cellular conditions, neurons increase their expression and/or secretion of CD200 and CX3CL1 and their binding to microglia through CD200R and CX3CR1 induces an alternative activation of microglia (Wong, 2013). In addition, CD200 and CX3CL1 have been shown to suppress both IL6 and TNF- α production in

microglia (Zujovic et al., 2000; Cardona et al., 2006; Pabon et al., 2011; Wong, 2013). We detected elevation in CX3CL1 expression following ethanol exposure in neonates and this increase in CX3CL1 level was progressively normalized following prolonged exposure. Interestingly, decrease of CX3CL1 correlated with the increase of both IL6 and TNF- α in neonates. This finding suggest that initial ethanol induced activation of microglia may be mediated by CX3CL1 following acute ethanol exposure, resulting in suppressed inflammatory signaling. Therefore, reduction of CX3CL1 expression may result in deregulation of microglial activation and result in increased inflammatory signaling following prolonged ethanol exposure. This hypothesis can be confirmed by quantifying microglial activation and its cytokine production upon neonatal ethanol exposure in CX3CR1-deficient mice.

5.2 ETHANOL INDUCED ALTERATIONS IN HPA AXIS

Prenatal alcohol exposure can result in severe problems in cognition, communication, learning, memory and behavior in affected patients (Hellemans et al., 2010). Mounting evidence from epidemiological and clinical studies demonstrate that fetal alcohol exposure can also results in milder cognitive, neuropsychological and behavioral deficits even in the absence of more severe problems associated FASD (Hellemans et al., 2010; Kilgour and Chudley, 2012). Milder effects of prenatal ethanol exposure include hyperactivity, poor attention span, impaired habitation, impulsivity, lack of inhibition and poor sensitivity to social cues in FASD patients (Hellemans et al., 2008; Hellemans et al., 2010). Prenatal and postnatal rodent models of FASD display similarly cognitive and behavioral abnormalities such as impaired learning and memory and hyper responsitivity to stressors (Weinberg et al., 2008; Hunt et al., 2009; Twari and Chopra, 2011).

Alterations in HPA axis has been implicated in many FASD related neuropsychological and behavioral abnormalities (Hellemans et al., 2008; Hellemans et al., 2010; Weinberg et al., 2010). We demonstrate for the first time that prolonged ethanol exposure during the neonatal period results in hyper responsiveness to acute restraint stress in adult males. In addition, we report that neonatal ethanol exposure alters the stress response curve to acute restraint stress. AF animals displayed a shift in the peak of CORT release in response to acute restraint stress suggesting abnormalities in HPA axis regulation. Transplantation of differentiated BEP neurons into the PVN not only significantly reduced stress response in AF animals, but also restored normal curve response to acute restraint stress.

Given that BEP transplantation only elevates BEP levels in the CNS without changing plasma BEP and attenuates the HPA hyper response in AF animals (Boyadjieva et al., 2009), the reduction of BEP expressing neurons may contribute to the CNS deregulation of the HPA activation observed in AF animals. In addition to its established role in CRH release regulation in the PVN, BEP producing POMC neurons may also play a key regulatory role in inflammatory signaling in the PVN. This regulation could take place through the suppression of local immune cell activation, including microglia (Figure 29). The interaction between BEP and microglia has yet to be described. We have begun to investigate the effects of BEP in stress mediated microglial activation following neonatal ethanol exposure. Supplemental experiments will be necessary to perform in order to confirm the possible regulation of microglial activation through BEP binding. BEP expressing neurons secrete BEP in response to acute challenge such as acute ethanol exposure in culture (Agapito et al., 2010). Therefore, we can test the interaction of BEP and microglia in primary cultures. Conditioned media from stimulation of pure in vitro differentiated BEP neurons will be used to test changes in activation of pure microglia cultures (Representation of proposed experiment; Figure 32). BEP actions will be confirmed using immunoneutralization of BEP.



FIGURE 32. PROPOSED EXPERIMENT TESTING THE REGULATION OF MICROGLIAL ACTIVATION BY BEP CONDITIONED MEDIA

Experimental Design. Pure Microglia and BEP neuronal culture will be stimulated by acute ethanol exposure. Following ethanol induced activation, microglia will be cultures with conditioned media from BEP neurons containing BEP and activation will be assessed by $CD11b^+$ gene and protein expression. Subset of microglia will be assessed without ethanol exposure to correct for potential effects of culture media. Abbreviation: EtOH.

We also observed a hyper response following immune challenge by LPS exposure as quantified by elevated CORT and ACTH plasma levels in AF animals compared to controls. This finding is in accordance with our previous report that neonatal ethanol exposure results in hyper response to immune challenge in adulthood as quantified by the increase in CRH mRNA expression in the PVN following LPS exposure (Sarkar et al., 2007).

Changes in HPA responsiveness to stressors in adulthood could result from various alterations throughout the HPA axis. Exposure to damaging factors such as ethanol during the developmental period could alter stress response in adulthood by permanently destroying pathways important for proper regulation of stress activation (Seckl and Meaney, 2004; Kapoor et al., 2006; Weinberg et al., 2008). In our postnatal model, we observed a proportional response between CORT and ACTH in AF animals comparable to controls following both acute restraint

stress and LPS exposure. Although ethanol induced changes in the HPA pathways may still be possible, we did not detect functional alterations in neither pituitary or adrenal cortex in response to stressors.

Alternatively, neonatal exposure to toxins including alcohol could re-program immune function to change their response to subsequent stress stimuli (either psychological or immune) through the exaggerated pro-inflammatory cytokine production in response to stress (Sliwowska et al., 2010; Bilbo, 2013). Interestingly, we found that minocycline pre-treatment during neonatal ethanol exposure prevents hyper response to LPS in adulthood as quantified by CORT and ACTH plasma levels. Moreover, increased expression of the pro-inflammatory cytokine TNF- α and elevation in activated microglia number were observed in the PVN following both acute restraint stress and LPS challenge in AF animals compared to controls, even in the presence of high CORT levels. Taken together, this data suggest that initial microglial activation during developmental period alters HPA axis responsiveness to subsequent stimuli in adulthood, possibly through changes in microglia sensitivity.

Importantly, this effect was only seen following prolonged ethanol exposure. Given the fact the intermittent binge drinking is more likely in non-alcoholic women during pregnancy as opposed to persistent binge drinking episodes (Nulman et al., 2004; Henderson et al., 2004; Sayal et al., 2009), it would be interesting to determine if these effects depend on the number of ethanol induced CNS insults or mainly due the maintained activation of microglia from continuous stimulation and release of inflammatory markers in response to ethanol exposure. These two paradigms can easily be distinguished by observing the immediate and long term effects of intermittent ethanol exposure on neonates (Representative Model, Figure 33).



FIGURE 33. EXPERIMENTAL MODEL OF INTERMITTENT ETHANOL EXPOSURE DURING DEVELOPMENT

(A) Representative model of intermittent ethanol exposure in neonates. Exposure to ethanol consists of two doses on each day by intubation of ethanol containing milk formula. Each day of exposure is followed by a 24 hours period of ethanol abstinence.(B) Representative model of intermittent neonatal ethanol exposure followed by behavioral assessment in adulthood. Abbreviations: EtOH, Ethanol; PD, Postnatal Day.

5.3 ETHANOL INDUCED ALTERATIONS IN MICROGLIA SENSITIVITY

Microglia have been shown to be sensitive to subsequent stimuli following large or chronic challenges (Crews et al., 2011; Qin et al., 2012; Barrientos et al., 2012; Bilbo, 2013; Schwarz and Bilbo, 2013). Moreover, the age of the individual appears to be a critical factor for the degree of microglia alterations following large activation (Barrientos et al., 2012; Bilbo, 2013). Emerging evidence suggest that changes in microglial response to subsequent stimuli may be a result of altered expression of surface protein and receptors responsible for the amplification of inflammatory signaling (Henry et al., 2008; Crews et al., 2013; Wong, 2013). Because of their

abilities to elicit innate immune activation through endogenous and exogenous ligands, Pattern recognition receptors (PRRs) have been discussed as candidates for increased inflammatory signaling in neurodegenerative diseases (Lucin and Wyss-Coray, 2009; Cribbs et al., 2012; Crews et al., 2013). Amongst all PRRs, Toll-like receptors (TLRs) are the best characterized (Qian and Cao, 2012).

TLRs are innate immune receptors that initiate downstream signaling pathways upon binding of pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs) and alarmins, molecules that can signal cell and tissue damage (Bianchi, 2007; Okun et al., 2011). Upon binding of these ligands, TLRs activate inflammatory signaling through MyD88-dependent and -independent pathways and recruit downstream kinases and transcription factors such as MAPK and NFkB to initiate inflammatory signaling (Kielian, 2009; Downes and Crack, 2010). There are currently 12 identified TLR family members in rodents (Qian and Cao, 2012). Although TLR 1-9 gene transcripts can be detected in rodent microglia (Kumar, 2013), only TLR1, 2, 3, 4 and 9 are constitutively expressed at the protein level (Lee et al., 2013).

Activation of TLR signaling has not only been shown to be important for induction of inflammatory responses, but has been demonstrated to be necessary for proper CNS development and repair following CNS damage (Larsen et al, 2007; Downes and Crack, 2010; Okun et al., 2011). Due to the potential damage of chronic inflammatory signaling, regulation of TLR activation is critical to maintain CNS integrity (Drexler and Foxwell, 2009; Ospelt and Gay, 2009; Downes and Crack, 2010). Dysregulation of TLR has been implicated in various CNS pathologies including neuroinflammation caused by age related microglial priming and chronic alcoholism (Lucin and Wyss-Coray, 2009; Buchanan et al., 2010; Cribbs et al., 2012; Crews et al., 2013).

Ethanol can induce TLR activation in the CNS through Myd88-dependent and – independent pathways (Alfonso-Loeches et al., 2010; Pascual et al., 2011; Crews et al., 2013). Moreover, ethanol activates microglial inflammatory signaling mainly through the activation of TLR2 and TLR4 (Fernandez-Lizarbe et al., 2009, Fernandez-Lizarbe et al., 2013; Crews et al., 2013). Most importantly, TLR2 and TLR4 activation have been shown to be crucial for ethanol induced neuroinflammatory damage and ethanol induced anxiety-like behaviors in adult rodents following chronic ethanol consumption (Alfonso-Loeches et al., 2010; Pascual et al., 2011). Permanent increases in TLR expression has been suggested to contribute to the long term CNS alterations caused by chronic ethanol exposure in adults (Crews et al., 2013).

Increased neuroinflammation through the activation of TLR receptors may also contribute to ethanol induced neurodegeneration and alterations in behavior following neonatal ethanol exposure. Immature microglia express TLR1-9 mRNA as early as embryonic day 13 (Kaul et al., 2012), however the protein expression of these are extremely low during the first postnatal week (Okun et al., 2011; Hichey et al., 2011). TLR2 and TLR4 expression has been shown to gradually increase from early embryonic stages into adulthood (Okun et al., 2011). Interestingly, LPS mediated activation of TLR4 during the first postnatal week increases TLR4 expression in the developing brain, leading to altered responses to subsequent stimuli (Hickey et al., 2011) suggesting that TLR transcripts can be translated in the presence of a stimulus.

Neuroinflammation and damage can be prevented with minocycline treatment. Minocycline pre-treatment has been shown to suppress LPS induced TLR2 and TLR4 signaling in microglia (Henry et al., 2008; Bastos et al., 2013), as well as prevent elevation of TNF- α levels in the brain (Zhou et al., 2006). Taken together, these finding suggest that minocycline may partly prevents microglial activation through the attenuation of TLR signaling.

Therefore, we postulate that microglia programming could result from increased TLR2 and TLR4 expression caused by prolonged neonatal ethanol exposure. Since TLR transcripts are expressed in neonates, ethanol could serve as a stimulus to induce translation of TLR2 and TLR4 mRNA following prolonged exposure. Furthermore, initial TLR activation could lead to amplified TLR expression on immature microglia. Given that microglia are long lived innate immune cells, microglia with greater number of TLRs may augment their responses to subsequent stimuli due to increased ligand-receptor binding (Representative model, Figure 34).



FIGURE 34. MODEL OF NEONATAL ETHANOL EXPOSURE REPROGRAMMING OF MICROGLIA THROUGH INCREASES OF TLR EXPRESSION.

(A) Ad libitum adult animals express normal number of TLR 2 and TLR4 on the surface of microglia and elicit proper activation in the presence of a stimulus. (B) Neonatal ethanol exposure may lead to increased TLR 2 and TLR4 expression on microglia resulting in exaggerated or abnormal activation in the presence of subsequent stimuli.

5.4 FUTURE DIRECTIONS

Consistent with our hypothesis, minocycline pre-treatment may prevent hyper response to subsequent stressors in adulthood by preventing initial ethanol induced TLR signaling in ethanol exposed animals and in turn, prevent enhanced expression of TLR2 and TLR4. The following experiments will be performed to test this hypothesis.

First, we will quantify TLR2 and TLR4 expression in neonatal ethanol exposed adult males at basal and following acute restraint stress and/or LPS challenge. If significant increases in TLR2 and/or TLR4 expression are detected in AF animals compared to controls, TLR2 and TLR4

mRNA and protein expression will be quantification in neonates following prolonged ethanol exposure. The quantification of both mRNA and protein expression is necessary because the transcription of TLRs may be up-regulated in microglia following prolonged neonatal ethanol exposure but may not be translated until presented with a subsequent stimulus. This effect is seen after LPS preconditioning of microglia in neonates where TLR4 increases its expression following subsequent hypoxia stimulus (Hickey et al., 2011). If differences are observed in AF neonates compared to controls, we will confirm the involvement of TLR signaling in microglia programming following ethanol exposure using TLR inhibitors. Various TLR agonists and antagonists have been described (Lee et al., 2013; Kumar, 2013), however, TLR2 seems to be the most promiscuous of all TLRs (Fernandez-Lizarbe et al., 2013), and may be difficult to completely inhibit. Therefore, if no suitable antagonist or inhibitor can be found, the use of TLR2^{-/-} and TLR4^{-/-} knockout mice may be required.

Finally, the identification of endogenous ligands will be necessary to confirm this mechanism. There is no evidence that ethanol itself binds to TLR2 or TLR4. However, it is well established that ethanol can induce the production and secretion of DAMPs and alarmins from neuronal cells upon exposure (Alfonso-Loeches and Guerri, 2011). Several TLR endogenous ligands have been identified (Table 3) (Lee et al., 2013). Amongst these, the alarmin High mobility group box 1 (HMGB1) has been demonstrated to have a role in ethanol induced neurodegeneration and behavioral deficits of adolescent and adult rodents (Qin and Crews, 2012; Vetreno and Crews, 2012; Crews et al., 2013). Moreover, neutralization of HMGB1 was shown to suppress ethanol induced neuroinflammation (Crews et al., 2013). Therefore, HMGB1 may contribute to ethanol induced CNS damage and alteration in neonates.

TLRs	Endogenouse ligands
TLR2	HSP60, 70, Gp96 HMGB1 β-defensin3 Surfactant protein A, D Eosinophil-derived neurotoxin Gangliosides Serum amyloid A Hyaluronic acid fragment
TLR3	mRNA small interfering RNA
TLR4	HSP60, 70, 22, Gp96 HMGB1 Fibrinogen Fibronectin extra domain A Tenascin-C Surfactant protein A, D β-defensin2 S100A8, 9 (MRP8, 14) Neutrophil elastase Lactoferrin Gangliosides Serum amyloid A Oxidised LDL Saturated fatty acids Hyaluronic acid fragment Heparan sulfate
TLR7	ssRNA
TLR8	ssRNA
TLR9	Chromatin-IgG complexes DNA immune complexes

Table 3. IDENTIFIED ENDOGENOUS TLR LIGANDS.

The endogenous TLR ligand HMGB is a known agonist of TLR2 and TLR4. Adaptation of Table 1 from Lee et al., 2013.

5.5 FINAL REMARKS

In conclusion, this work provides important insights into the effects of both immediate and long term effects of ethanol induced microglial activation during third trimester gestation. In our model, ethanol induced microglial activation leads to long term alterations in microglial responses to subsequent stimuli that contribute to changes in HPA response in adulthood. Further investigation in the permanent alterations of surface receptors expression on microglia following prolonged neonatal ethanol exposure may yield novel insights into the role of microglia programming in HPA axis alterations.

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