ROLE OF BETA-ENDORPHIN IN CONTROL OF STRESS AND

CANCER PROGRESSION IN FETAL ALCOHOL EXPOSED RATS

by

CHANGQING ZHANG

A dissertation submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

In partial fulfillment of the requirements

For the degree of

Doctor of Philosophy

Graduate Program in Endocrinology and Animal Biosciences

Written under the direction of

Dipak K. Sarkar, Ph.D

And approved by

_____

_____

_____

_____

New Brunswick, New Jersey

October, 2013

ABSTRACT OF THE DISSERTATION

Role of beta-endorphin in Control of Stress and Cancer Progression

in Fetal Alcohol Exposed Rats

By CHANGQING ZHANG

Dissertation Director:

Dr. Dipak K. Sarkar

Fetal alcohol exposure causes a series of defects in the animals, including hyper-reactivity to stress, impaired immune function, and increased susceptibility to mammary cancer. Production of β-endorphin (β-EP), the neuronal peptide that inhibits stress axis, is also reduced in the hypothalamus of fetal alcohol exposed animals. Therefore, we hypothesized that the loss of β-EP neurons in the hypothalamus may be the leading cause of the hyper-reactivity to stress, which then compromises the immune system and leads to increased progression of mammary cancer. Furthermore, we proposed that enhancement of β-EP neuronal function will have therapeutic effects on treating neoplastic diseases.

To test this possibility, we developed two methods that increase the β-EP neuronal numbers in the hypothalamus: 1) transplantation of *in vitro* differentiated β-EP neurons into the paraventricular nucleus of the hypothalamus; 2) injection of nanosphere-carried cAMP activating reagents into the third ventricle. Both of these methods could significantly increase the β-EP peptide level in the hypothalamus and significantly decrease the activity of stress axis. By using these methods, we could increase the activities of innate immune function and inhibit the growth of carcinogen-induced mammary cancer as well as the metastasis of a mammary carcinoma cell line.

These beneficial effects on immune function and cancer development caused by increased β-EP production could be reversed by treating the animals with naloxone (opioid receptor antagonist), metaproterenol (β-adrenergic receptor agonist), and methyllycaconitine (α7 nicotine acetylcholine receptor antagonist). After β-EP neuronal transplantation, nude rats that lack T-cell-mediated adaptive immune reactivity also showed significant resistance to mammary cancer cell metastasis. These data indicate that the effect of β-EP on mammary cancer growth works through activation of the opioid receptor, inhibition of the sympathetic nervous system, activation of the parasympathetic nervous system, and activation of the innate immune function.

In conclusion, measures that activate β-EP neurons in the hypothalamus could inhibit activity of the stress axis, increase innate immune function and inhibit mammary cancer growth and metastasis in both normal and fetal alcohol exposed animals. β-EP neuronal loss in fetal alcohol exposed patients may be an important cause of the problems with stress, immune function and cancer.

DEDICATION

I dedicate this work to my family for being supportive, inspiring and loving. My dad, a professor in math and computer science, set a good example for me on pursuing the fun of learning and thinking. My mom raised my interest on biological science and science related to daily life by encouraging me to keep any creatures as pets, to grow any plant in the pot, and to find out the hidden reasons of life phenomena. My husband has been taking care of me ever since I came abroad. Life wouldn't have been so easy and comforting without him and his love.

I'd also like to dedicate this work to my beloved grandfather-in-law and my aunt, whose life were taken by cancer. They are the fundamental motivations for me to choose to work with this cancer-related project.

Lastly, I will also dedicate this thesis work to Dr. Dmitry Govorko, who has been a good colleague and friend.

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

α7 nAChR	α7 nicotine acetylcholine receptor
ACTH	Adrenocorticotropin Hormone
AD	ad libitum
AF	alcohol-fed
ANS	Autonomic Nervous System
AVP	Arginine Vasopressin
β-EP	beta-endorphin
BBB	Blood Brain Barrier
BrdU	Bromodeoxyuridine
CAM	Cell Adhesion Molecule
cAMP	Cyclic Adenosine Monophosphate
CNS	Central Nervous System
Con A	Concanavalin A
CREB	cAMP-responsive element binding protein
CRH	Corticotropin-Releasing Hormone
dbcAMP	dibutyryl cAMP
ELISA	Enzyme-linked Immunosorbent Assay
EMT	Epithelial-Mesenchymal Transition
FAE	Fetal Alcohol Exposure
FAS	Fetal Alcohol Syndrome
FASD	Fetal Alcohol Spectrum Disorders

GD	Gestational day
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor
GR	Glucocorticoid Receptor
H&E	Hematoxylin and Eosin
HPA	Hypothalamic-Pituitary-Adrenal
IEG	Immediate early gene
IFN-γ	Interferon-γ
IgG	Immunoglobulin G
IL	Interleukin
ISO	isoproterenol
LC-NE	locus ceruleus norepinephrine
LPH	lipotropin
LPS	Lipopolysaccharide
MET	Metaproterenol
MHC	Major Histocompatibility Complex
MLA	Methyllycaconitine
MLN	Mesenteric lymph node
MMP	Matrix Metalloproteinase
MNU	N-methyl-N-nitrosourea
MP	Metaproterenol
MR	Mineralocorticoid Receptor
MSH	Melanocyte-stimulating hormone
NIAAA	National Institute on Alcohol Abuse and Alcoholism

NK	Natural Killer
NSC	Neuronal Stem Cell
PACAP	Pituitary adenylate cyclase-activating peptide
PBMC	Peripheral Blood Mononuclear Cells
PD	Postnatal Day
PF	pair-fed
PKA	Protein Kinase A
PLGA	poly(lactic-co-glycolic acid)
POMC	proopiomelanocortin
PSNS	Parasympathetic nervous system
PVN	paraventricular nucleus
Rb	Retinoblastoma
SD	Sprague Dawley
SNS	Sympathetic Nervous System
SS	Serum Supplement
SVZ	subventricular zone
TGF-β	Transforming growth factor-β
Th	Helper T cells
TH	Tyrosine Hydroxylase
TNF	Tumor Necrosis Factor
VEGF	Vascular Endothelial Growth Factor

CHAPTER 1

1   **Chapter 1: Review of the literatures**

1.1   **Introduction**

Prenatal alcohol exposure produces a range of adverse outcomes in offspring, collectively referred to as fetal alcohol spectrum disorders (FASDs). FASD patients show a wide range of deficits in growth, anatomy, behavior and cognition [1]. It is estimated that in the United States and Europe the prevalence rate of FASDs is up to 10 in 1000 live births [2-3]. The most severely affected children on the spectrum show a series of anomalies including growth retardation, malformations on the face and permanent central nervous system damage, which are called fetal alcohol syndrome (FAS) [4]. Fetal alcohol exposure is the leading known cause of mental retardation in the western world. In addition to morphology and behavioral abnormalities, many studies have connected fetal alcohol exposure (FAE) with stress hyper-responsiveness [5-6] and decreased immune function [7], that possibly causing higher incidence of bacterial infection [8], and higher lifetime risk of different types of cancers [9-12]. The mechanism by which fetal alcohol exposure causes these detrimental changes is not clear.

As a teratogen, alcohol could cause all kinds of complex changes through physical, physiological, hormonal and metabolic disturbances on the developing fetus. There are recent evidences that the altered stress response in fetal alcohol exposed animals may play an important role in the immune function deficiency and increased cancer susceptibility. It is well-established that fetal alcohol exposed animals have hyper-responsive stress axis [13]. Stress is a state of altered homeostasis under threatened situations, and by which the body copes with intrinsic or extrinsic adverse forces. The body and mind react to stress by activating a complex and strictly regulated repertoire of central nervous system and peripheral adaptive responses. Stress can significantly affect many aspects of the body's immune systems. For example, higher levels of stress were shown to be associated with decreased Natural Killer (NK) cell lysis activity, T cell population, lymphocyte

proliferation following infection, and interferon-γ (IFN-γ) levels [14]. These factors are known to be important components of immunity against cancer [15-16].

Additionally, it was reported that chronic high levels of stress can increase carcinogenesis in rat models [17]. Therefore manipulations to control stress response and reduce stress level may be a promising treatment to increase immunity and fight against neoplastic diseases in fetal alcohol exposed animals. β-endorphin (β-EP), an endogenous opioid polypeptide produced by hypothalamic neurons, pituitary endocrine cells and immune cells, is known to have the ability to inhibit stress hormone production, produce analgesia and a feeling of well-being [18-19]. It was found that fetal alcohol exposed rats have reduced β-EP production [20]. Lower numbers of β-EP neurons or peptide production have been also found in brains of patients with schizophrenia, depression, and obese patients [20-22], and these pathological conditions were correlated with higher incidences of cancers and infections [23-25]. Therefore it may be that the decrease in β-EP levels is involved in the mechanism by which fetal alcohol exposure increases susceptibility to cancer. Hence, the possibility arises that controlling stress level through manipulations to increase β-EP production may have the potential to be used as a promising treatment against cancer and diseases caused by immune-deficiency in fetal alcohol exposed animals and patients.

1.2 Overview of fetal alcohol spectrum disorders

1.2.1 FASD: epidemiology and definition

Alcoholic beverages that contain ethyl alcohol (ethanol) are natural products of fermentation of many fruits and have been produced and consumed by humans all around the world since pre-historical times. Alcoholic beverages are often an important part of different cultures, from hunter-gatherer people to nation-states. People from the ancient times took alcohol for its euphoric and recreational effect, for inspiration, or for addition of flavors to diets. Ethanol, the

principal psychoactive constituent in alcoholic beverages, has both psychoactive effect which produces a state known as intoxication and also depressant effects on the central nervous system (CNS). It has a complex mode of action and affects multiple systems in the brain and the body.

In the adult body, ethanol is metabolized into acetaldehyde by alcohol dehydrogenase, then converted to acetic acid by aldehyde dehydrogenase, and finally bound to coenzyme A (CoA) to form acetyl CoA, an intermediate common with glucose and fatty acid metabolism that can be used for energy generation in the citric acid cycle or for biosynthesis. With these steps, alcohol is metabolized within hours, leaving little effect afterwards. However, when consumed by pregnant female mammals, like pregnant women, ethanol acts as a teratogen that causes birth defects in their babies. Alcohol's ability to cause birth defects was recognized more than three decades ago by U.S. researchers, when they first noticed that children born to alcoholic mothers showed similar patterns of craniofacial, limb, and cardiovascular defects associated with prenatal-onset growth deficiency and developmental delay [26]. Alcohol is now the leading known environmental teratogen, and the leading non-genetic cause of mental retardation in the western world [27], which causes devastating consequences broadly termed fetal alcohol effects (FAEs).

Currently, there is a high rate of alcohol consumption in pregnant women and women that can possibly get pregnant. According to the statistics of NIAAA (National Institute on Alcohol Abuse and Alcoholism), 28.8% of American women reported binge drinking, which is consumption of more than 4 drinks within 2 hours, and 22.98% of American women drank drinking alcohol at least once a week in the past year. In a 1981 advisory, the U.S. Surgeon General suggested that pregnant women should limit their alcohol intake. Despite the educational efforts to raise awareness of the detrimental effects of alcohol on the fetus, there are still approximately 7.6% of pregnant women in the U.S. using alcohol, and 1.4% of these women reported binge drinking, according to the CDC (Centers for Disease Control and Prevention) research in 2012 [28]. Population-based data from the Behavioral Risk Factor Surveillance System (BRFSS) for the

years 1991~2005 showed that more than 12% of women who are not using contraception and are at risk of becoming pregnant are drinking at levels that exceed 7 drinks per week or more than 4 drinks per occasion [29]. Many women don't recognize their pregnancy until 4~6 weeks gestation [30]. Therefore many women may drink before they recognize that they are pregnant, and place their fetus at high risk of developmental disorders. The prevalence of FAS in the U.S. is 0.5~2.0 cases per 1000 live births. It may be more common in other parts of the world. For example, the incidence of FAS in South Africa, where heavy drinking prevails, is 60 cases per 1000 live births. It is estimated that the prevalence of FASD is about three times as frequent as FAS and its incidence is 1 per 100 live births in the US [31].

FAS is one of the most serious consequences of heavy drinking during pregnancy, first described by Jones and Smith in 1973 [26]. FAS is a devastating birth defect characterized by craniofacial malformations, neurological and motor deficits, intrauterine growth retardation, learning disabilities, and behavioral and social deficits. The diagnosis of FAS requires: 1) maternal alcohol consumption, 2) characteristic facial dysmorphologies, 3) growth retardation, and 4) central nervous system (CNS) neurodevelopmental abnormalities [32]. The characteristic facial dysmorphologies include short palpebral fissures, thin upper lip, flat midface, and smooth philtrum [33]. Growth retardation includes low birth weight and lack of weight gain over time, with disproportional low weight to height ratio. Due to ethanol's effect on migration of neuronal and glial progenitor cells, the most dramatic and devastating changes that occurs in FAS patients are abnormalities in the CNS. Children with prenatal alcohol exposure show characteristic patterns of brain abnormalities including microcephaly, enlarged ventricles, disorganization of cerebral brain matter, hydrocephalus, reduced white matter volume relative to gray matter, reduced volumes of parietal lobe, cerebellum and basal ganglia, and abnormalities of corpus callosum [34-36].

FAS and FASD both describe the adverse effects of FAE on the brain during the critical period of embryonic development while FAS is the more severe form of these two. The severity of phenotypes between the two depends on the amount, duration, period and frequency of exposure during fetal development. FASD or alcohol-related neurodevelopmental disorder are umbrella terms that describe a range of abnormalities resulting from prenatal alcohol exposure, include FAS, alcohol-related birth defects, and alcohol-related neurodevelopmental disorders [32].

Imaging and neurobehavioral research in individuals with FAS and FASD reveals that some brain regions appear to be more sensitive to prenatal alcohol exposure compared to other areas. Particularly vulnerable regions include the frontal cortex, hippocampus, corpus callosum, and components of cerebellum, including the anterior vermis. In addition to structural changes, FASD patients show a range of typical behavior abnormalities that are related to the affected brain structures. A study of FAS patients using Life History Interview suggested that these patients had major problems with adaptive behavior. Characteristic behavior patterns in adolescents and adults with a history of prenatal alcohol exposure are disrupted school experiences, trouble with the law, inappropriate sexual behaviors, alcohol or drug use problems, and problem with confinement [37]. Mood disorders, major depressive disorder and bipolar disorder are also common in these patients [38]. Attention deficit, hyperactivity and overstimulation disorders are the common comorbid conditions in individuals with FASD.

1.2.2 Mechanism that causes FASD

The mechanism by which ethanol alters fetal development is complex. Ethanol is a simple, small molecule (CH_3-CH_2-OH). Because of the hydroxyl group on one end of this molecule, it is able to form hydrogen bonds, and is miscible with water. Because of the carbon chain on the other end, it is also miscible with many organic solvents and therefore able to pass through cell membranes without the need of specific transport mechanism. In the bodies of pregnant women, ethanol

readily crosses the placenta and enters the fetal circulation. The main pathway for maternal ethanol metabolism is via the enzyme alcohol dehydrogenase in the liver, which converts alcohol to acetaldehyde by oxidation. Alcohol affects the developing fetus both directly as a teratogen and indirectly by its metabolites and its effects on maternal physiology [32].

The metabolite of ethanol, acetaldehyde, is a highly unstable compound and can quickly form free radicals that are highly toxic and can therefore result in damage to embryonic neural crest cells and lead to severe birth defects. Ethanol itself can also disrupt neuronal cell-cell adhesion, by directly affecting neural cell adhesion molecules (CAMs) and by influencing the gene expression of CAMs such as L1 [39-40]. Cell-cell adhesion is critical during the neurodevelopmental period, and its disruption results in neurodevelopmental disorders including microcephaly and mental retardation [41].

Ethanol is known to cause apoptotic cell death of neurons, which occurs by several different mechanisms. Our lab was one of the first to show that ethanol neurotoxicity on fetal neurons during early neuronal differentiation involves an apoptotic process [42]. Our lab further demonstrated that ethanol stimulates apoptotic cell death of fetal rat hypothalamic β-EP neurons by increasing caspase-3 activity, suppression of cAMP, and activation of transforming growth factor-β1 (TGF-β1)-linked signals [42-43]. Fetal alcohol exposure also increases the production of reactive oxygen species, cytochrome c and caspase 3 in the brain [44-45], which are components of the intrinsic pathway of apoptosis. Studies with whole embryo culture showed that ethanol-induced superoxide anion and lipid peroxidation caused excessive cell death, which then lead to failure of the anterior neural tube to close [46]. Ethanol can easily cross the cell membranes and damage both cellular and mitochondrial DNA [47], also leading to apoptosis. Ethanol has also been shown to interfere with the cell signaling pathway involving insulin-like growth factor I receptor, thus preventing the progression of cell cycle and inhibiting cell proliferation, as well as leading to apoptosis [48]. Apoptosis of cranial neural crest cells is

believed to cause the distinctive facial features seen in FAS [49]. Cell death caused by fetal alcohol exposure particularly involves serotonergic neurons [50].

Maternal alcohol intake also affects the fetus at the level of the placenta. Ethanol inhibits transport of critical cofactors across the placenta, such as biotin [51] and vitamin B6 [52]. Ethanol also causes decreased levels of nitric oxide in the placental villi [53] and induces rapid vasoconstriction in human placenta [54], thereby decreasing oxygen delivery. Ethanol was also found to disturb the development and structure of the placenta, in terms of decreased placenta weight, decreased content of glycogen, altered cytoarchitecture of the labyrinthine zone, and formation of large cysts [55].

The earliest stages of life are periods of great vulnerability to adverse effects of alcohol. Embryonic and fetal developments are characterized by rapid, but well-synchronized patterns of gene expression, which makes the embryo/fetus particularly vulnerable to harm from alcohol. The vulnerability to alcohol exposure usually occurs during the first and second trimester of pregnancy, which correlate with the timing of essential developmental processes such as neurogenesis, cell migration, cell adhesion and synaptogenesis [56-57]. Alcohol exposure during the third trimester of pregnancy targets particularly the cerebellum, the hippocampus and the prefrontal cortex, and causes abnormalities such as cognitive and intellectual malfunctions [56]. An *in vitro* study using human trophoblast cells showed that first-trimester ethanol-induced production of the cytokines granulocyte colony-stimulating factor (GM-CSF) and interleukin-6 (IL6), and altered cytokine levels during early gestation may have an adverse effect on the development of the feal immune system [58]. In humans, late-third trimester maternal alcohol exposure results in decreased fetal breathing movements for 3 hours after the consumption, although the lasting consequence is unknown [59].

Some of the detrimental effects of prenatal alcohol exposure may be mediated by epigenetic changes, which are stable but potentially reversible alterations in a cell's genetic information that result in changes in gene expression but does not involve changes in the underlying DNA sequence. The development and function of the CNS requires accurate gene transcription control under the influence of different environmental signals. Alcohol exposure induces epigenetic modifications such as changes in DNA methylation [60-61], histone modifications [62-63] and small noncoding RNAs [64]. The metabolite of ethanol, acetaldehyde, was shown to inhibit DNA methyltransferase activity and cause hypomethylation of fetal DNA in mice exposed to ethanol during fetal development [60]. Increased H3K9/18 acetylation of histone was detected in alcohol-exposed fetuses, and this change could alter gene expression and induce apoptosis [63]. Four miRNAs, miR9, miR21, miR153 and miR335 were found to be suppressed by ethanol in neuronal stem cells (NSCs), and may cause abnormalities in apoptosis, proliferation and differentiation of NSCs [64]. Abnormalities in these epigenetic modulations caused by fetal alcohol exposure may cause significant changes in gene expression and cellular functions, and lead to defects of neuronal structures and cognitive functions [65].

Current treatments that aim to counteract the effects of prenatal alcohol exposure largely mirror the proposed mechanisms by which alcohol induces damage. Targets have been found that disrupt ethanol's inhibition of L1 CAM in cell adhesion *in vitro* [66]. Antioxidants, including superoxide dismutase [46], vitamin C [67], vitamin E [68], and green tea extract [69] have been shown to counter the oxidative stress of ethanol in animal models. Supplementation of vitamin A/retinoic acid, which plays important roles in neurogenesis and was shown to be disturbed by fetal alcohol exposure, significantly decreased the degree of birth defects normally caused by ethanol in rodent models [70]. Prevention of ethanol-induced damage to serotonergic neurons in fetal rats has been demonstrated by serotonin agonists, such as buspirone and ipsapirone [71-72]. Methyl donors such as choline and folate were also used to balance the hypomethylation caused by fetal alcohol

exposure, and were shown to be effective in mitigating the adverse effects of fetal alcohol exposure on development, behavior, memory, attention and learning performance in animal models [70, 73-75].

Treatment of fetal alcohol exposure before birth would be the most effective. Once the baby is born, the dysmorphology and CNS structural abnormalities caused by prenatal alcohol exposure are irreversible. Therefore postnatal therapies for FASD patients mostly focus on management of associated neuropsychological behaviors and prevention of comorbid conditions. However, it was revealed recently that the postnatal neuronal system still has plasticity that gives rise to new neurons and connections in response to stimuli in the environment. Therefore therapies may be devised to stimulate brain functions and reverse the defected behaviors in fetal alcohol exposed patients. For example, voluntary aerobic physical exercise was shown to have therapeutic potential for FASD by stimulating brain-derived neurotrophic factor, increasing cell proliferation, neuronal maturation and cell survival [76], and reversing the depression-like behaviors [77], cognitive impairment and hyper-activity [78] in rat model. However, prevention is still the best option for dealing with fetal alcohol exposure. More effort should be made to raise the awareness about FASD among the general public.

1.3   Fetal alcohol spectrum disorders and hyper-stress response, immunity and cancer

1.3.1   Stress response in FASD

In the last two decades, a group of studies revealed that disturbance of fetal growth is linked with behavioral and mental health outcomes later in life. FASDs are disorders that occur to the embryo when a pregnant woman ingests alcohol during pregnancy, causing not only a wide range of deficits in growth, anatomy and cognition, but also numerous secondary disabilities including behavior changes, depression and anxiety disorders [6]. Clinical reports showed that patients with FASDs had high frequencies of psychiatric problems such as somatoform disorder, substance

dependence or abuse disorders, paranoid, passive-aggressive, antisocial, and dependent personality disorders [79-80]. Clinical study with a group of adolescents with FASD also showed that they have higher incidence of self-harm related consultation such as attempts of suicide [81]. In summary, patients with FASD experience mental health problems, and these problems may result from the abnormal brain structure, neurotransmitter production, and neuroendocrine regulation including stress regulation.

Stress reactivity is defined as the extent to which a person is likely to respond to a stressful event or environment. It serves as a link between environment and psychological and somatic distress, and has been shown to be a vulnerability factor for physical [82-83] and mental disease [84-85]. (Fig. 1 [86]) The stress axis is highly susceptible to programming during fetal and neonatal development [87]. The concept "fetal programming" states that the effect of environmental or nongenetic factors during sensitive periods of organ development may cause long-lasting changes in the structure and functioning of these organs later in life and influence the risk for chronic diseases. The hypothalamic-pituitary-adrenal (HPA) axis is especially vulnerable to the programming effect of fetal alcohol exposure, which manifests as abnormal responsiveness to stress in adulthood. A study by Jacobson et al. reported that high levels of maternal drinking at conception and during pregnancy were associated with higher basal and post-stress cortisol levels in infants at 13 months of age [5].
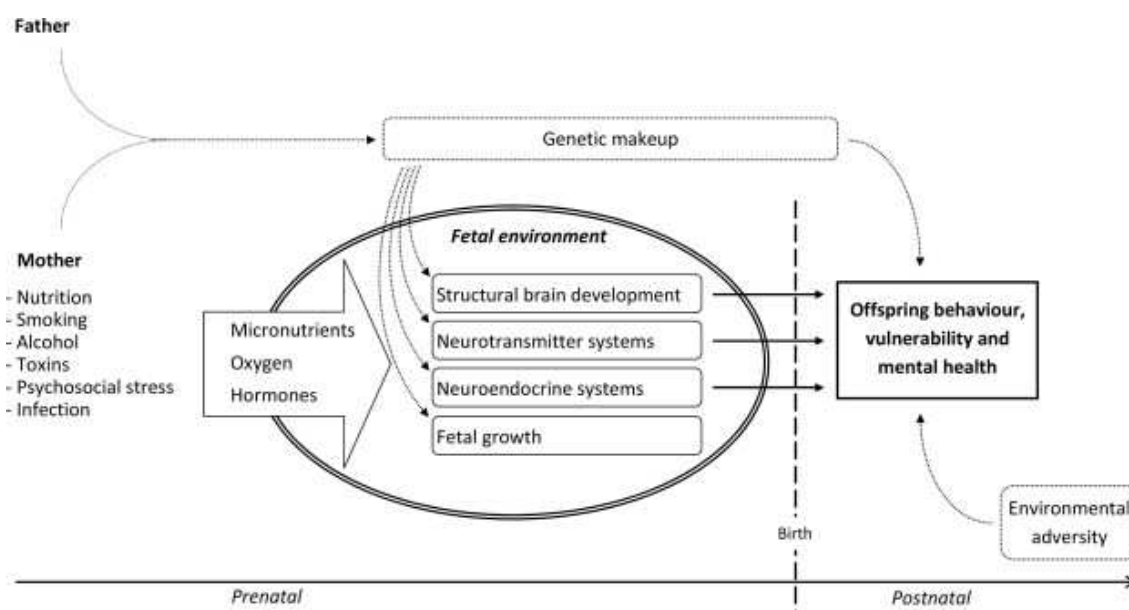
Figure 1: Impact of environmental factors on fetal programming.

Adapted from Schlotz 2009 [86].

Animal models are widely used for the study of the effects of fetal alcohol exposure on stress. Alcohol exposure in utero programs the fetal HPA axis therefore increasing the HPA tone throughout life. Studies by Weinberg and others have shown that prenatal alcohol exposure results in HPA hyperactivity and altered regulation of HPA comparable to the model system of depression. Their data suggest that fetal alcohol exposed rats show hyper-responsiveness to stressors, increased HPA drive, and deficits in HPA feedback regulation [6, 88-89]. Both males and females also show increased immediate early gene (IEG) and corticotropin-releasing hormone (CRH) mRNA levels following stress [90], as well as deficient habituation to repeated restraint stress [91]. Fetal alcohol exposure alters levels of adrenocorticotropin hormone (ACTH) and corticosterone [91-95] as well as beta-endorphins [96] in response to different stressors. Fetal reprogramming of the HPA axis and sensitizing of the organism to subsequent stressful experiences by alcohol may be the underlying mechanism of these physiological and behavioral

changes. In general, maternal alcohol consumption reprograms the fetal HPA axis such that the HPA tone is increased throughout life.

In addition to the HPA axis, the autonomic nervous system (ANS) is also altered by fetal alcohol exposure. Fetal alcohol exposed mice were shown to have altered noradrenergic synaptic transmission, including enhanced norepinephrine turnover and a reduction in norepinephrine levels and β-adrenergic receptor density in the thymus and spleen [97], which may indicate an abnormal regulatory effect of the sympathetic nervous system (SNS) on immune function.

The effect of fetal alcohol exposure on stress response in the offspring may be related to alcohol-induced endocrine imbalances. Alcohol readily crosses the placenta as a small molecule, therefore easily affecting the developing endocrine organs in the fetus. Alcohol can also induce changes in maternal endocrine functions, and disrupt the hormonal interactions between the pregnant female and the developing fetus, alter the normal hormone balance, and indirectly affect the development of fetal metabolic, physiological and neuroendocrine function. Alcohol exerts both direct and indirect effects on many hormone systems, including the adrenal, gonadal, and thyroid axes, as well as aldosterone, growth hormone, parathyroid hormone, calcitonin, insulin, and glucagon [13]. Alcohol also causes changes in peripheral hormone metabolism and hormone binding. Whether alcohol-induced endocrine imbalances and physiological changes actually contribute to the etiology of FASD is unknown, but it is certainly a possibility [98]. Alcohol-induced changes in maternal metabolic and/or endocrine function can affect the female's ability to maintain a successful pregnancy, resulting in miscarriage or, if the fetus is carried to term, possible congenital defects.

Hyper-active stress axis in fetal alcohol exposed animals may be an important aspect that induces the changes in stress hormone levels that occur in the pregnant female. Data using animal models indicate that maternal alcohol consumption increases maternal adrenal weights, basal

corticosterone levels, the corticosterone response to stress, and the corticosterone stress increment, compared with those in pair-fed and control groups [93, 99]. These changes occur as early as Day 11 of pregnancy, persist throughout gestation, may increase as gestation progresses, and occur even with low concentrations of ethanol in the diet [99-100]. The stimulatory effect of ethanol on HPA axis activity can extend through parturition, even when alcohol administration is discontinued before parturition [94]. The pregnant female and her fetus constitute an interrelated functional unit. Therefore ethanol induced alterations in maternal HPA activity may have significant effect on fetal HPA development. Maternal corticosterone crosses the placenta [101], resulting in suppression of endogenous fetal HPA activity. At the same time, ethanol crosses the placenta and directly activates the fetal HPA axis. These hormonal changes caused by indirect and direct ethanol exposure affect the brain organization of the developing fetus and cause permanent imprint on the stress axis activity [102].

Studies were done in an attempt to test the hypothesis that increased maternal glucocorticoid levels play a role in the hyper-responsiveness of HPA axis in fetal alcohol exposed offspring. However, the results are difficult to explain. It has been shown that adrenalectomy of the pregnant dam has no effect on the increased glucocorticoid responses to restraint stress in ethanol exposed offspring, but reverses the increased immobility of fetal alcohol exposed offspring in the forced swim test of depressive behavior [103]. Another study showed that treatment of adrenalectomized dams with glucocorticoid does not mimic the effect of prenatal ethanol exposure on HPA axis activity in the offspring [104]. However, maternal adrenalectomy reversed the stimulatory effect of prenatal ethanol on pituitary proopiomelanocortin (POMC, the precursor polypeptide of ACTH) mRNA levels observed in ethanol exposed offspring [105].These findings together suggest that increased maternal glucocorticoid levels may not be the primary mediator of increased stress responsiveness under some stress paradigms in fetal alcohol exposed offspring.

Increased HPA activity could result from increased secretion of secretagogues (CRH, arginine vasopressin (AVP) and ACTH), increased pituitary and/or adrenal responsiveness to these secretagogues, increased drive to the hypothalamus, and deficits in feedback regulation of HPA activity. In fact, it may be a result of the combination of all these factors. Evidence suggests that fetal alcohol exposure enhances stimulatory inputs or drive to the paraventricular nucleus (PVN) of the hypothalamus. Lee et al. demonstrated that 21 day old male and female rat pups exposed to ethanol during the second week of gestation, exhibit increased basal levels of CRH mRNA in the PVN and ACTH secretion [106]. They also reported that fetal ethanol exposed adult animals exhibit significantly increased IEGs c-fos and NGFI-B mRNA levels in the PVN of fetal alcohol exposed rats after physical and immune stressor treatment, suggesting an increased neuronal activity of hypothalamic CRH perikarya [90]. CRF but not AVP hnRNA levels were also significantly increased in PVN of these animals [90]. After adrenalectomy, fetal alcohol exposed animals showed higher basal plasma ACTH, PVN CRH mRNA, and decreased pituitary CRH R1 mRNA levels compared to adrenalectomized control animals, indicating a lack of negative feedback regulation of the HPA axis independent of corticosterone [107-108]. The hippocampal mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) mRNA were also altered in both male and female animals exposed to ethanol during fetal period [13]. Fetal alcohol exposed rats also showed resistance to dexamethasone suppression of glucocorticoid level during stress [109]. These data from Weinberg's lab indicate that fetal alcohol exposure alters the negative feedback mechanism of the stress axis.

Embryonic exposure to ethanol reduces the number of neurons in various parts of the central nervous system including the hypothalamus [13]. The number of β-EP neurons was found to be reduced in the hypothalamus of fetal alcohol exposed animals throughout life [110-111]. Plasma levels of β-EP were also found to be reduced in children of alcoholic parents [112]. β-EP is an inhibitor of CRH and down-regulates the activity of the stress axis. These animals also showed

increased response of the hypothalamic CRH mRNA to LPS challenge, as well as incompetent ability to respond to exogenous β-EP to alter the LPS-induced CRH mRNA levels [110], indicating an altered negative feedback regulatory mechanism. Therefore we hypothesize that the hyperactivity of the stress axis in fetal alcohol exposed rats is caused by decreased production of β-EP hormone and decreased regulatory function of β-EP on the stress axis. Our lab has shown that by putting back *in vitro* generated β-EP neurons into the hypothalamus, the hyper-stress axis was inhibited [113], indicating a causal relationship between decreased hypothalamic β-EP and hyper-active stress axis in fetal alcohol exposed animals. The detailed mechanism of FAE on β-EP and POMC gene expression will be discussed in the following sections.

1.3.2   Immune function in FASD

Another system that appears to be affected by ethanol exposure during fetal life is the immune system [7]. Impairments in immune competence of children with FAS have been described in both innate and adaptive immunity. The innate immune system, also known as non-specific immune system, forms the first line defense against common pathogens and abnormally growing cells in a non-specific manner. Adaptive immunity, or acquired immune system, is MHC (major histocompatibility complex) restricted and consists of two parts: cellular and humoral, mediated by T and B lymphocytes, respectively. Children prenatally exposed to alcohol often show defects in host defense and propensity to infections, such as meningitis, pneumonia, recurrent otitis media, astroenteritis, and sepsis, as well as urinary tract and frequent upper respiratory tract infection [8, 114]. These children also have lower cell counts of eosinophils and neutrophils, decreased circulating E-rosette-forming lymphocytes, reduced mitogen-stimulated proliferative responses by peripheral blood leukocytes, and hypo-c-globulinemia [8]. These defects were found not to be

related to the degree of postnatal growth retardation nor to degree of malnutrition and do not alleviate with increasing age.

In accordance with clinical observations, research using animal models also reveals negative impacts of fetal alcohol exposure on immune function of the offspring. Prenatal ethanol exposure using animal models was shown to negatively affect lymphoid tissue development, immune cell function, humoral immunity and cytokine secretion [115]. Activity of NK cells, which is the first line of immune defense against infection and cancer, is suppressed in fetal alcohol exposed animals [113]. Steven and coworkers showed that the immune response of fetal ethanol exposed neonates to the intestinal parasite *Trichinella spiralis* revealed a diminished capacity to respond to the pathogens in these animals, which was demonstrated by an increased intestinal worm count [116-117]. These animals were found to have lower immunoglobulin G (IgG) antibody as a sign of diminished primary immune response, and reduced IgM and IgG antibodies as a sign of diminished secondary immune response. They also had lower serum interleukin-2 (IL-2) and tumor necrosis factor (TNF) levels after the infection challenge. Mesenteric lymph node (MLN) cells showed reduced proliferation responses to antigen and Con A in these fetal alcohol exposed animals [117].

Fetal alcohol exposure alters thymus development in fetuses of rodents as indicated both by histological immaturity in structure [118] and by cell number and proliferative response to concanavalin A (Con A) [119]. The highly specific microenvironment of the thymus provided by epithelial and mesenchymal cells is important for T cell maturation. Therefore alteration of thymus structure may have detrimental effects on thymus function and immune response. In fetal alcohol exposed 21-day-old rats, T lymphocyte proliferation stimulated by the mitogen Con A was significantly lower in both spleen and thymus [120]. Thymic cell counts and total number of immature $CD4^+CD8^+$ cells were decreased in fetal alcohol exposed young adult male rats, and immature $CD8^+TCR^+$ and $CD8^+CD45RC^+$ thymocytes were reduced in these animals [121]. The

period before parturition is considered to be critical in lymphocyte maturation in rodents, because adult-like precursor cells as well as mature accessory cells populate the thymus by embryonic days 19~20 [122-124].

In vitro study also confirms the adverse effect of ethanol on the development of the thymus. Total cell numbers and percentage of immature fetal thymocytes (CD4$^+$CD8$^+$) were found to be decreased in a dose responsive manner in ethanol-treated fetal mice thymus cultures, and cells with mature phenotypes (CD4$^+$CD8$^-$, IL-2 receptor negative, and γ/δ-T-cell receptor positive) were increased [125]. These decreases may have been resulted from accelerated apoptosis of fetal thymocytes and increased percentage of noncycling thymocytes [125].

The spleen is an important immune organ that hosts large numbers of mature immune cells, called splenocytes. Fetal alcohol exposure decreased the numbers of Thy1.2$^+$, CD4$^+$, CD8$^+$, and IgG$^+$ splenocytes, and this effect was also shown in mice with early postnatal exposure to alcohol, which is equivalent to the third trimester gestation in humans [126-127]. Fetal alcohol exposed rats and mice showed decreased splenic T lymphocyte proliferative responses to mitogens (Con A or graft-to-host response) from adolescence to adulthood [97, 128-130], and this defect in T-cell proliferation was due to an inability of lymphoblasts to utilize exogenous IL-2 [128-129]. A group investigating the higher infection and death rate in fetal alcohol exposed non-human primates (macaca nemestrina) showed that these animals had decreased T cell proliferation response to tetanus toxoid, and decreased antigen-specific memory to tetanus toxoid vaccination [131]. Other than the affected cellular immunity mediated by T cells, humoral immunity appears to be less affected by fetal alcohol exposure.

The mechanism behind the immune deficiency is complex. It may at least be partially due to the abnormal stress regulation and CNS function in fetal alcohol exposed animals. The reciprocal interactions between the immune and the nervous system are necessary for a competent immune

response. Interactive effects between stress and fetal alcohol exposure on immune function were shown by studies of Giberson et al. Under stressed conditions, fetal alcohol exposed rats had a greater reduction in the number of pan T cells in the thymus and peripheral blood, and greater reduction of $CD4^+$ cells in the peripheral blood, comparing to unstressed fetal alcohol animals [95]. With cold stress, fetal alcohol exposed female rats showed significant differences in mitogen-induced lymphocyte proliferation compared with control animals [132]. A significant increase in thymic CRH and decrease in thymic POMC gene expression was observed on day 19 of gestation [133]. Fetal alcohol exposure suppressed the lymphocyte proliferative response to Con A in male rats from immature to young adult age, and this effect of fetal alcohol was reversed by maternal adrenalectomy [105]. Therefore maternal adrenal hormones participate in the immunosuppressive imprinting of the FAE fetus and are possible causes of lymphocyte dysfunction found in FAE offspring.

It was recently found that fetal alcohol exposed rats exhibit increased plasma levels of proinflammatory cytokines including IL-1, TNF-α and IL-6 following repeated stress [13]. Increased exposure to endogenous glucocorticoids throughout the lifespan can alter behavioral and physiological responsiveness and increase vulnerability to illnesses or disorders later in life. Alterations in immune function may be a consequence of altered HPA programming.

1.3.3 Cancer in FASD

In addition to an abnormal stress response and immune system, patients with FASD were also found to suffer from increased incidence of neoplastic diseases. As soon as FAS was defined three decades ago, clinical researchers started to pay attention to other side effects that accompanied FAS, and noticed an association between fetal alcohol exposure and childhood neoplasia [134]. Clinicians reported that there were many cases of children admitted to the hospital with FAS in conjunction with benign or malignant tumors, while the clinical cases found

didn't show uniformity as to tumor type. FAE children may develop mild hyperplasia such as tibial exostoses [135] as well as malignant cancer like embryonal rhabdomyosacoma of urinary bladder and prostate [136]. A case-control study showed some evidence of an increased risk of childhood acute myeloid leukemia in fetal alcohol exposed children [11]. Maternal consumption of alcohol during pregnancy was also found to be associated with development of testicular cancer in the sons [12].

Animal researches confirmed the observations in clinical studies, and showed that fetal alcohol exposure increase the incidence of tobacco-related pancreatic cancer [137], β-estradiol induced prolactinoma [138], and both carcinogen induced and gyngenetic mammary tumor growth [9-10, 139]. Our lab has also shown that fetal alcohol exposure increased the incidence of carcinogen-induced prostate cancer [140].

The increased incidence of tumor origenesis in fetal alcohol exposed offspring may be due to multiple factors, including: 1) defects in immune function, which leads to impaired immune surveillance; 2) abnormal hormone levels, such as stress hormones, which suppress immune function; and 3) altered organ development and gene expression in the affected tissue by developmental imprinting. Alcohol administration has been reported to increase circulating estradiol levels in pre-menopausal women [141]. Trichopoulos et al. raised the hypothesis that elevated exposure to estrogens in utero could possibly increase the lifetime risk of breast cancer by altering normal breast development [142]. Later on, this hypothesis was supported by the findings of a number of epidemiological studies [143]. Animal research also supported this hypothesis and showed that elevated in utero estradiol levels lead to altered mammary gland development and increased susceptibility to carcinogen-induced mammary tumors [9, 144]. Alcohol consumption also decreased the level of aromatase, and therefore decreasing testosterone levels in the pregnant moms, which is connected with higher incidence of testicular cancer in the male offspring [145]. It was also found that fetal alcohol exposed female offspring have increased

circulating E2 levels, which may serve as a factor that increases mammary cancer, prostate cancer and prolactinoma development [9]. Our lab has recently found that prostates of fetal alcohol exposed rats had increased levels of estrogen receptor (ER)-α, decreased levels of androgen receptor and decreased levels of circulating testosterone [140].

Abnormalities of immune function may be another factor that causes the increased tumor incidence in fetal alcohol exposed animals, since the immune system is important for cancer surveillance and elimination. Alterations in immune function and susceptibility to cancer may be one of the long-term consequences of a hyperactive stress axis. It was shown that immune deficiency caused by chronic alcohol exposure, such as decreases in NK cell activity and cytokine production can be reversed by intracranial administration of β-EP [146-147]. With the fact that β-EP acts as a negative control of the stress system, and that its production is decreased in fetal alcohol animals and their offspring, we hypothesize that by replenishing β-EP in the hypothalamus, we could reverse the hyper-activity of the stress axis, the defect of immune function, and the susceptibility to cancer in fetal alcohol exposed animals.

1.4   POMC neurons and fetal alcohol exposure

Proopiomelanocortin (POMC) is the common precursor polypeptide for the melanocortin-related peptides (ACTH/α-melanocyte-stimulating hormones (MSH), β-MSH, and γ-MSH) and the opioid β-endorphin [148]. The gene encoding POMC polypeptide is a member of the *opioid/orphanin* gene family [149], most likely derived from an ancestral opioid-coding gene in chordate [150], and is conserved across all jawed vertebrates [151-152]. The structure of the POMC gene is similar among all mammalian species [153], indicating that POMC-derived peptides have important roles in animal survival. In humans, the POMC gene resides in chromosome 2p23 [154], contains three exons and two introns, and spans 7665 base pairs (bp)

including two introns of 3708 and 2886 bp [155-156]. The POMC gene contains three different promoters, which regulate the differential transcription of POMC gene in different tissues. These promoters are embedded within a defined CpG island, and are methylated in normal non-expressing tissues, which is sufficient for silencing the expression. In tissues expressing POMC gene, the promoters are specifically unmethylated in order to allow the binding of the transcriptional factors [156].

POMC gene is expressed in the brain, pituitary, and periphery. In the brain, this gene is primarily expressed by neurons in the arcuate nucleus of the hypothalamus, the amygdala, the cortex, and the nucleus tractus solitaries of the brainstem [157-160]. In the pituitary, POMC mRNA were highly expressed in the anterior and neurointermediate lobes [157]. In the periphery, POMC expression was found in detectable quantities in the semen and testes [161], ovaries and placenta [162], peripheral mononuclear cells (PBMCs) [163], thymus [164],and some tumors [165].

The POMC polypeptide contains 241 amino acids, weighs 32 kDa, and can be cleaved into many biologically active neuropeptide hormones including ACTH, β-EP, lipotropins (LPHs) and MSHs by individual processing through a series of tissue-specific co- and post-translational modifications (Fig. 2 [159]). POMC is processed differentially in the pituitary: in the anterior lobe into ACTH and β-LPH, while in the intermediate lobe into α-MSH and β-EP [158]. In the hypothalamus, POMC is processed into β-EP, γ-lipotropin and α-MSH [166].
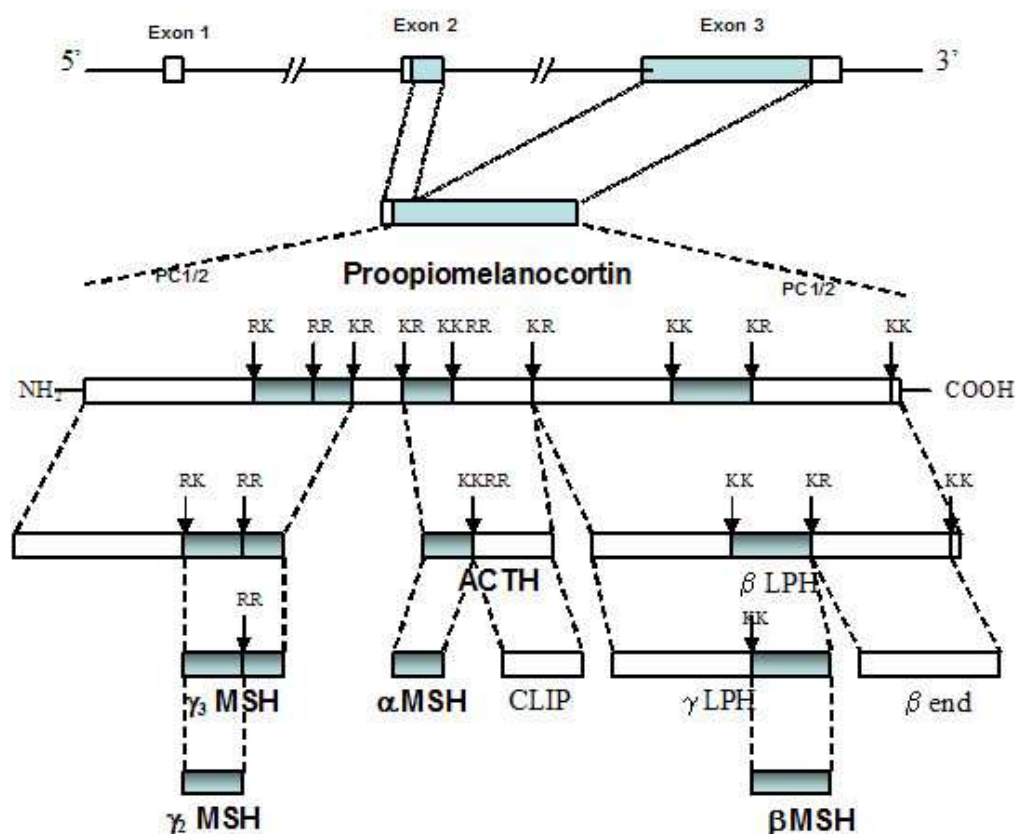
Figure 2: Gene structure and post-translational processing of proopiomelanocortin (POMC).

Adapted from Millington 2007 [159].

POMC gene plays an important role in the regulation of the HPA axis, adrenal development, depression, skin pigmentation and obesity. In the mediobasal hypothalamus, POMC expressing-neurons are located primarily in the arcuate nucleus, and β-EP is one of the primary products from these neurons. The transcription of POMC is stimulated by CRH [167], and β-EP in turn inhibit CRH production as a negative feedback [168]. In the anterior but not in the intermediate pituitary, glucocorticoids inhibit POMC gene transcription [169]. As discussed above, the HPA axis in fetal alcohol exposed animals is dysregulated, possibly due to a loss of negative feedback. Therefore, disturbance of POMC gene transcription may be an important cause of the hyper-reactive HPA axis in fetal alcohol exposed animals.

It has been found that the POMC system can be affected by fetal alcohol exposure. β-EP is one of the primary POMC-derived peptides in the hypothalamus and has been found to play a role in prenatal ethanol exposure induced growth retardation, behavioral abnormalities, central nervous system damage, and neuroendocrine abnormalities [170-172]. Studies showed that alcohol exposure altered energy regulatory genes. For example, fetal alcohol exposed adult rats showed increased expression of gluconeogenic genes, decreased glucose tolerance index, and insulin resistance [173-174]. POMC expressing cells, such as β-EP and α-MSH neurons in the hypothalamus, are critically involved in regulation of food intake and metabolism [175]. This hypothesized correlation is supported by findings of our lab showing that fetal alcohol exposure reduces POMC gene expression, especially β-EP peptide production in the hypothalamus [42-43, 110].

In the pituitary of fetal alcohol exposed rats, β-EP content in the pituitary is increased on postnatal day (PD) 4 in the whole pituitary and the anterior lobe, but decreased on PD8, 14 and 22 in the whole pituitary, anterior lobe, and the intermediate lobe [176]. It was also reported that fetal alcohol exposure decreased pituitary β-EP reaction to stressors such as ether and cold, and that hypothalamic CRH was increased, probably due to the lack of an inhibitory effect from β-EP [96]. Fetal alcohol exposure also blunted the response of pituitary β-EP release to IL-1β stimulation [177]. However, the plasma level of ACTH, also a product from the POMC gene, is increased in FAE animals, suggesting an increased ACTH production from the pituitary [111]. Although some data indicate that the total POMC mRNA levels weren't changed in the pituitary of fetal alcohol exposed animals [107], elevated plasma ACTH levels were observed in these animals [107].Therefore POMC gene regulation is complex and these experimental findings indicate that the effect of FAE may cause differential cleavage of POMC peptide that favors one of its cleavage products over another.

Besides the hypothalamus and pituitary, POMC gene expression in the peripheral organs, such as the thymus, is also altered by fetal alcohol exposure. FAE results in a significant increase in thymic CRH and a decrease in thymic POMC expression in the male fetus at embryonic day 19 [133]. Under normal conditions, expression of CRH stimulates POMC expression, and therefore POMC should increase as CRH increases in the thymus. However in the case of fetal alcohol exposed fetuses, the positive correlation between CRH and POMC is abolished [133]. POMC system in the thymus is a functional part of the microenvironment of this organ, as CRH and POMC derived peptide, including ACTH, β-EP, and α-MSH have been shown to be potent immunomodulators [178]. Therefore the modulation of POMC by fetal alcohol exposure in the thymus may contribute to the impaired immune function in fetal alcohol exposed children and animals.

The effect of alcohol exposure on β-EP production has been studied using *in vitro* models with neuronal cultures. Acute ethanol administration increases the release of β-EP in hypothalamic neuronal cultures, in the hypothalamus and the periphery, while high dose or chronic ethanol decreases its release [179-181]. Our lab has shown that treatment with a cAMP-elevating agent, forskolin, increases cAMP levels in hypothalamic cell culture, and this elevating effect is reduced by ethanol [182]. Therefore the stimulatory and adaptive response of β-EP neurons to ethanol may involve the cAMP system.

Our lab has done extensive work investigating the effect of fetal alcohol exposure on hypothalamic POMC gene expression and stress axis. We reported that fetal alcohol exposed animals have decreased hypothalamic POMC gene expression, decreased β-EP neuronal number and peptide production, and decreased NK cell function [20, 110-111, 113]. The decreased number of β-EP neurons in the arcuate nucleus of the hypothalamus may be due to decreased differentiation of NSCs into β-EP neurons during development, and increased apoptotic death of β-EP neurons [20, 110]. Since ethanol molecules easily cross the placenta, when the pregnant

female drinks alcohol neuronal cells in the developing fetal brain will be directly exposed to ethanol. We have shown that addition of ethanol to fetal rat hypothalamic cell cultures stimulates apoptotic cell death of β-EP neurons by increasing caspase-3 activity, suppression of cAMP, and activation of transforming growth factor-β1 (TGF-β1)-linked signals [43]. TGF-β1 has significant apoptotic action on mediobasal hypothalamic neuronal cells. The increased TGF-β1 in tissue is correlated with a reduction of retinoblastoma protein (Rb) phosphorylation in the arcuate nucleus, which then leads to an increase of cyclin dependent kinase inhibitor p27/kip level and decrease of cyclin dependent kinase 4 and cyclin D3 levels [20]. These factors interact with each other and lead to inhibition of cell proliferation and apoptotic neuronal death.

Out lab has also used a postnatal ethanol exposure rat model [110], which is equivalent to the third trimester pregnancy in humans, and showed that immediately after ethanol ingestion by the babies, there was a significant reduction in the expression of POMC and adenylyl cyclase mRNA and an incrased expression of TGF-β1-linked apoptotic genes in β-EP neurons in the acuate nucleus. These data indicate that fetal alcohol exposure causes apoptosis of β-EP neurons, possibly through a cAMP and TGF-β1 related pathway. Since microglia are the major inflammatory cells in the central nervous system, and they may be involved in the elevated pro-inflammatory cytokine production and fetal alcohol-induced neuronal death. We showed that ethanol's ability to induce apoptotic death of neurons was increased by the presence of ethanol-activated microglia-conditioned media, which contained increased levels of inflammatory cytokines including TNF-α [183].

In addition to a direct toxic effect of ethanol exposure on fetal brain tissue and neurons, ethanol-induced physiological changes may cause imprinting of the neuronal cells during the critical period of fetal development. Epigenetic modification is a highly possible imprinting mechanism for the POMC gene. Methylation of the promoter region is both sufficient and essential for the inhibition of POMC gene expression [156]. We have reported that the methylation of several

CpG dinucleotides in the proximal part of the POMC promoter region was increased in fetal alcohol exposed male and female rats [111] and that the effect was reversed by gestational choline supplementation [184], which serves as a methyl donor. These data indicate that the abnormalities in methylation status caused by ethanol may be a factor that imprints the abnormal stress regulation [111].

1.5   Stress and its mechanism

1.5.1   Definition of stress

Stress and related concepts can be traced as far back as written science and medicine. The first formal definition of stress didn't appear until 1973, when Hans Hugo Bruno Seyle, known as the 'father of stress', defined stress as "the non-specific response of the body to any demand imposed upon it" [185]. As described in Seyle's book *Stress in Health and Disease*, the detailed definition of stress in behavioral science is regarded as the perception of threat, with resulting anxiety, discomfort, emotional tension, and difficulty in adjustment. Stress can also be defined in terms of pure neuro-endocrinology. For example, Eugene Yates defined stress as any stimulus that will provoke the release of ACTH and glucocorticoids [186]. Similarly, sympathetic hormones like epinephrine/norepinephrine can also be employed as markers of stress, as defined by Walter Cannon [187]. Later on in 1992, Chrousos and Gold modified this definition by changing the term "non-specific" to the hypothesis that above a certain threshold intensity any stressor would elicit the "stress syndrome" [188]. These two scholars also raised the concept of "stress system", which is the effector of the stress syndrome. They concluded that the main components of the stress system are the corticotrophin-releasing hormone and locus ceruleus-norepinephrine/autonomic system and their peripheral effectors, the pituitary-adrenal axis, and the limbs of the autonomic system [188]. In general, stress can be defined as a real or interpreted

threat to the physiological or psychological integrity of an individual that results in an elevation of the adrenal glucocorticoids and/or catecholamine levels.

Stress can occur in a variety of forms, physical or psychological, acute or chronic. Acute stress has been defined as stress that lasts for a period of minutes to hours, and chronic stress defined as stress that persists for at least several hours per day for weeks or months [189]. The stimulus input, or stressor, refers to an event that elicits a non-selective response (endocrine, behavioral, autonomic) that targets multiple tissues and organs in response to a challenge to homeostasis. Messages of "stress" are communicated through synapses in the brain and translated into increases in circulating stress hormones which prepare every cell in the body for a time of compromise [190]. Diverse interoceptive and exteroceptive stress stimuli, arriving through afferent inputs, increase the activity of neuroendocrine cells in the paraventricular nucleus (PVN) of the hypothalamus. Starting from the PVN, stress is known to cause the release of several stress hormones—particularly glucocorticoids through activation of the HPA axis and catecholamines through the sympathetic nervous system [14].

1.5.2   Stress pathways and regulation

Response to stress is conducted by the stress system, which is located both in the central nervous system (CNS) and the periphery. The stress system receives and integrates a diversity of cognitive, emotional, neurosensory, and peripheral somatic signals that arrive through distinct pathways. There are at least two major categories of stressors, physical and psychological, and the brain reacts to them differently [191]. Physical stressors produce actual disturbances of physiological status that hit specific homeostatic mechanisms. Examples of these stressors are haemorrhage, trauma, cold or infection. On the other hand, psychological stressors are stimuli that mentally threaten the individual's current or anticipated state, such as social conflict, aversive environment, examinations or deadlines, and predator-related cues.

Physical and psychological stressors elicit different brain circuits. Physical stressors preferentially recruit the brainstem and hypothalamic regions and activate the central nucleus of the amygdale [192-193]. By contrast, psychological stressors primarily engage stress mediators in brain regions that subserve emotion (the amygdala and the prefrontal cortex), learning and memory (the hippocampus) and decision making (the prefrontal cortex), and preferentially activate the medial nucleus of the amygdale [191, 194-196]. Both of these two categories of stressors showed similar strength in activation of the HPA axis in terms of glucocorticoid secretion, which is one of the endpoints of the stress response [191]. PVN receives extensive affect information from brain stem nuclei, sensory afferents, circumventricular organs and other hypothalamic areas [197]. Medullary catecholamine cells in the brainstem contribute to HPA axis responses to both physical and psychological stressors, but only in the case of physical stressor does this involve a direct input to PVN CRH cells [198-199].

Neuroendocrine cells in the PVN are the target of synaptic inputs from a number of brain structures including the limbic brain, and in particular the medial nucleus of the amygdala [191]. In response to a stressor, the 41-amino acid peptide corticotropin-releasing hormone (CRH) and the 9-amino acid peptide arginine-vasopressin (AVP) are secreted into the hypophyseal portal system from activated neurons of the paraventricular nuclei (PVN) in the hypothalamus. AVP of magnocellular neuron origin is also secreted into the hypophyseal portal system via collateral fibers and the systemic circulation via the posterior pituitary [200-201]. The CRH and AVP stimulate release of ACTH from the anterior pituitary into the general bloodstream, which results in secretion of glucocorticoids from the zona fasciculate of the adrenal cortex [202-203]. AVP, although showing a potent synergistic effect with CRH on ACTH secretion, has very little ACTH secretagogue activity on its own [204]. Other factors, including angeotensin II, various cytokines (e.g. IL-1β), and lipid mediators of inflammation are secreted in response to different types of stressors, and act on the HPA axis and potentiate its activity.

Glucocorticoids are the final effectors of the HPA axis and play an essential role in maintaining basal and stress-related homeostasis [205]. These hormones are pleiotropic, and exert their effects through their intracellular receptors which are ubiquitously distributed [206]. The effects of these hormones can be broadly classified into two major categories: immunological and metabolic. Immunological effects include up-regulation of anti-inflammatory cytokines and down-regulation of pro-inflammatory cytokines, as well as inhibition of development of immune cells such as T lymphocytes. Metabolic effects include stimulation of gluconeogenesis, mobilization of amino acids, inhibition of glucose uptake in muscle and adipose tissue, and stimulation of fat breakdown in adipose tissue. Glucocorticoids exert negative feedback on the secretion of CRH and ACTH through type II glucocorticoid receptors, therefore limiting the duration and intensity of the tissue exposure to stress hormones, in order to minimize the catabolic, lipogenic, anti-reproductive, and immune-suppressive effects of these hormones [207].

Parallel to the HPA axis, the autonomic nervous system (ANS) provides a pathway for rapid response to stressors, the fight-or-flight response, engaging the sympathetic nervous system (SNS) and withdrawing the parasympathetic nervous system (PSNS), thereby enacting physiological changes. The ANS controls a wide range of functions, including performance of cardiovascular, respiratory, gastrointestinal, renal endocrine, and other systems. Sympathetic innervation of peripheral organs is derived from the efferent pre-ganglionic fibers, whose cell bodies lie in the intermediolateral column of the spinal cord. These nerves synapse in the bilateral chain of sympathetic ganglia with post-ganglionic sympathetic neurons. Activation of the sympathetic nervous system results in secretion of acetylcholine from the pre-ganglionic sympathetic fibers in the adrenal medulla, and causes the release of epinephrine and norepinephrine into blood circulation. Post-ganglionic neurons also innervate many peripheral organs such as the smooth muscle of the vasculature, the heart, skeletal muscles, kidney, gut, and fat. Release of glucocorticoid can also be regulated by the autonomic nerves of the adrenal cortex [208].

The HPA axis and the ANS are not two isolated pathways. Instead, they interact with each other and share many regulatory factors. Reciprocal neuronal connections exist between the CRH and noradrenergic neurons of the central stress system. CRH stimulates norepinephrine through CRH type 1 receptor, while norepinephrine stimulates CRH through α_1- and α_2-noradrenergic receptors [209-211]. Both the CRH and the noradrenergic neurons receive stimulatory innervations from the serotoninergic and cholinergic systems [212-213], and inhibitory inputs from the γ-aminobutyric acid (GABA), opioid peptide neuronal systems of the brain [211, 214-215], and the end-product of the HPA axis-glucocorticoids [211, 216].

The stress system interacts with three other major CNS components: the mesocorticolimbic dopaminergic system, the amygdale-hippocampus complex, and the hypothalamic arcuate nucleus POMC neuronal system [207]. These systems are activated during stress and in turn influence the activity of the stress system. Both the mesocortical and mesolimbic components of the dopaminergic system are innervated by PVN CRH neurons and the locus ceruleus norepinephrine (LC-NE) system, and are activated during stress [217-218]. Both of these systems consist of dopaminergic neurons of the ventral tegmentum. The mesocortical system innervates the prefrontal cortex, while the mesolimbic system innervates the nucleus accumbens. The amygdale-hippocampus complex is activated during stress primarily by ascending catecholaminergic neurons originating in the brainstem, by glucocorticoids, and by emotional stressors which are generated in the amygdale [219]. LC-NE and the CRH/AVP producing neurons reciprocally innervate and are innervated by opioid peptide (β-EP)-producing neurons of the arcuate nucleus in the hypothalamus. Activation of the stress system stimulates hypothalamic POMC-derived peptides, such as α-MSH and β-EP, which reciprocally inhibit the activity of HPA axis and SNS, and produce analgesia through projections to the hind brain and spinal cord [207].

Our lab has focused largely on the effect of the hypothalamic opioid peptide, β-EP, on its effects in stress regulation. β-endorphin is an endogenous opioid polypeptide compound, a cleavage product of POMC which is also the precursor hormone for ACTH. β-EP is produced by the pituitary gland and the hypothalamus in vertebrates during exercise, excitement, pain, consumption of spicy food and orgasm, and it resembles the opiates in its abilities to produce analgesia and a feeling of well-being [18, 220]. β-EP neuronal cell bodies are primarily localized in the arcuate nuclei of the hypothalamus, and its terminals are distributed throughout the central nervous system, including many areas of the hypothalamus and limbic system [221]. β-endorphin is released into blood from the pituitary gland and into the CNS from hypothalamic neurons.

β-EP is known to directly inhibit the stress system. β-EP neurons innervate CRH neurons in the PVN and inhibit CRH release [211, 222], while naltrexone, a μ-opioid receptor antagonist, increases it [223]. Another μ-opioid receptor antagonist, Naloxone, also increases arterial pressure during bleeding by increasing glucocorticoid levels [224], which is a sign of an activated stress axis. During stress, secretion of CRH and catecholamines stimulates secretion of hypothalamic β-EP and other POMC-derived peptides, which in turn inhibit the activity of these two pathways [225].

Abnormalities in β-EP neuronal function are correlated with different types of emotional and physical diseases. For example, lower numbers of β-EP neurons have been reported in brains of patients with schizophrenia, depression, and fetal alcohol syndrome [21, 113, 226]. Reduced β-EP production was observed in obese patients [22], and a higher incidence of cancers and infections was found to correlate with these pathological conditions [24]. Hence, it appears that hypothalamic β-EP inhibits CRH secretion, and by doing so, regulates sympathetic outflow to the spleen and positively regulates immune function.

1.6   Impact of stress on immune and cancer

1.6.1   Stress and immune

The idea that psychological states can affect the outcome of human disease has been accepted for a long, long time. Aristotle, Hippocrates, and the other Ancients were already aware of stress and its adverse effects. It has been known that stress compromises immune function and may have some relationship to cancer. Human body and mind response to stress is tightly regulated. During acute stress, stress hormones can help enhance immune function by informing the immune system about impending challenges that may be imposed by a stressor. If reaction to stress is inadequate or excessive and/or prolonged, it may affect many physiological functions such as growth, metabolism, circulation, reproduction, and inflammatory/immune response [207].

Stress has many adverse effects on immune function by both suppressing some immune activities and increasing other immune activities. For example, stress suppresses key immune factors that are important for fighting against infection and cancer, while enhancing some immune functions that may exacerbate asthma, and allergic, autoimmune and inflammatory diseases. In humans, stressed and depressed patients had an overall leucocytosis (increased leukocyte count, frequently a sign of inflammation), mild reduction in absolute NK cell counts and functions, reduction in relative T cell proportions and functions, marginal increases in the ratio of CD4$^+$ and CD8$^+$ cell populations, higher concentrations of circulating neutrophils, reduced mitogen-stimulated lymphocyte proliferation and neurophil phagocytosis, and reduced and changed monocyte activities [227-229]. Social stress, such as conjugal bereavement, separation and divorce are associated with health risk in women [230]. Women who had separated from their husbands within the previous years had poorer immune function than did sociodemographically matched married women, with significantly poorer proliferation in response to mitogens and lower proportions of NK cells and helper T cells [231]. Stress was also associated with increased prevalence of tuberculosis and vulnerability to common cold virus [14].

Animal studies have also shown that environmental stressors affect immunological functions. As early as three decades ago, Monjan et al. have shown that spleen cells isolated from mice exposed to daily sound stress had a reduced ability to respond to test mitogens [232]. Stressful conditions can greatly suppress the immune response of blood and spleen lymphocytes in animals, including T-cell mitogenesis, production of IgG2a, NK cell activity, and production of IL-2 and IFN-γ [233].

Immune function is highly susceptible to changes in hormone levels. Immune cells, such as lymphocytes and macrophages, possess various kinds of receptors, and are regulated by molecules including neurotransmitters (norepinephrine, serotonin, dopamine, and acetylcholine), neuropeptides (enkephalines, substance P, vasoactive intestinal peptide, CRH, and neuropeptide Y), neurohormones (growth hormone, ACTH, and prolactin), and adrenal hormones (glucocorticoids and epinephrine). The fact that the neuroendocrine and immune systems share common signal molecules and receptors suggests that the brain has an immunoregulatory role [234-235]. On the other hand, it has been recently found that the cytokines IL-1, TNF-α, IFN-α, and IFN-γ secreted from activated immune cells can in turn change the function of the HPA axis, indicating a cross talk between stress regulation and the immune system [236]. Hormones produced in reaction to stress have detrimental effects on immune functions, including reduced NK cell activity, lymphocyte population, lymphocyte proliferation, antibody production and reactivation of latent viral infections [14]. The magnitude and duration of stress-induced elevation in catecholamine and glucocorticoid hormones can have significant effects on immune cell distribution and function [237-239].

Cytokines are soluble mediators released by immune cells at the periphery and by astrocytes and microglia in the CNS, which operate within a complex network and stimulate a series of immune and physiological reactions. Glucocorticoids, norepinephrine and epinephrine inhibit the production of human IL-12 by antigen-presenting cells such as monocytes, macrophages and

dendritic cells. Because IL-12 and TNF-α promote type 1 helper T cell (Th1) responses and cellular immunity, whereas IL-10 suppresses both the production of IL-12 and Th1 activity and stimulates Th2 and humoral immune responses, the stress hormones seem to cause a selective suppression of Th1 responses.

Chronic stress can significantly affect the body's immune system. For example, higher levels of stress were shown to be associated with increased sympathetic activity and decreases in NK cell lysis activity and IFN-γ levels [240-241]. These effects are caused at least partially by glucocorticoids. Glucocorticoids, which exist at high concentrations during stress, have important immunosuppressive effects on the functions of lymphocytes and macrophages, and might affect their circulation patterns [242]. It is known that glucocorticoids can modulate the transcription of many cytokines. They suppress the pro-inflammatory cytokines IL-1, IL-2, IL-6, IL-8, IL-11, IL-12, TNF-α, IFN-γ, and GM-CSF, and decrease the effects of some inflammatory molecules on various target tissues, while up-regulating the anti-inflammatory cytokines IL-4 and IL-10 [243]. They also suppress maturation, differentiation and proliferation of immune cells, including innate immune cells, T cells, and B cells [243].

Catecholamines released from the SNS during stress, especially epinephrine and norepinephrine, also have many immunomodulatory effects and are released from the nerve terminals in the vicinity of immune cells [244-245]. Catecholamines are reported to be immunosuppressive. Norepinephrine (NE) disturbs inflammatory cytokine networks by inhibiting the production of immune-enhancing cytokines like IL-12 and TNF-α, and by up-regulating the production of inhibitory cytokines like IL-10 and TGF-β [243]. Stress experiments suggest that the plasma concentration of epinephrine is inversely related to specific immune functions of lymphocytes and monocytes [233]. Our lab has shown that the β-adrenergic agonist isoproterenol (ISO) and β2-selective agonist metaproterenol (MP), which mimics the effect of activated SNS, both inhibit

the protein and mRNA levels of perforin, granzyme B and IFN-γ, and the inhibition effect of MP is blocked by β-antagonist nadolol [241]. Norepinephrine inhibits NK cell cytolytic activity [241].

NK cells are a subset of lymphocytes, providing first line defense against viral infection, tumor growth and metastasis by their unique cytolytic action [246]. Cytolytic activity of NK cells involves the synergistic action of pore-forming protein perforin and the serine protease granzyme B to cause apoptosis of target cells [247]. Among the HPA hormones, glucocorticoid and CRH have been shown to be potent inhibitors of NK cell activity *in vitro* and *in vivo*. β-adrenergic receptors were found on NK cells [248]. Hypothalamic CRH inhibits NK activity and IFN-γ production through activation of SNS, which causes release of catecholamines in spleen and activation of β-adrenergic receptor on NK cells [249].

β-EP is a neuropeptide that has the ability to suppress the stress reactivity of both the HPA axis and the autonomic system. Both central and peripheral β-EPs have profound positive effects on immune function. In the CNS, β-EP is known to bind to δ- and μ-opioid receptors and modulate the neurotransmission in sympathetic neurons to alter NK cell cytolytic functions in the spleen. Our lab has reported that intracranial administration of β-EP or δ-opioid receptor-specific agonist increases protein and mRNA levels of cytolytic factors in NK cells, including granzyme B, perforin and interferon-γ (IFN-γ) [146, 250]. NK cells utilize these cytokines to not only control viral infections in a non-cytolytic way, but also activate dendritic cells and helper T-cells to shape the specific immune response. Since the β-EP in the CNS is primarily produced from the acuate nucleus in the hypothalamus, this finding indicates the immune-enhancing role of hypothalamic β-EP (Fig. 3). Central infusion of β-EP or δ-opioid receptor–specific agonists also promotes the lymphocytes' proliferative response to mitogens, and this effect is blocked by naltrindol, which is a δ-opioid receptor antagonist [250-251]. In the periphery, opioid receptors are also present on lymphocytes [252], and β-EP is known to increase NK cell cytolytic function in splenocytes and peripheral blood mononuclear cells (PBMCs) through direct contact [253-254].
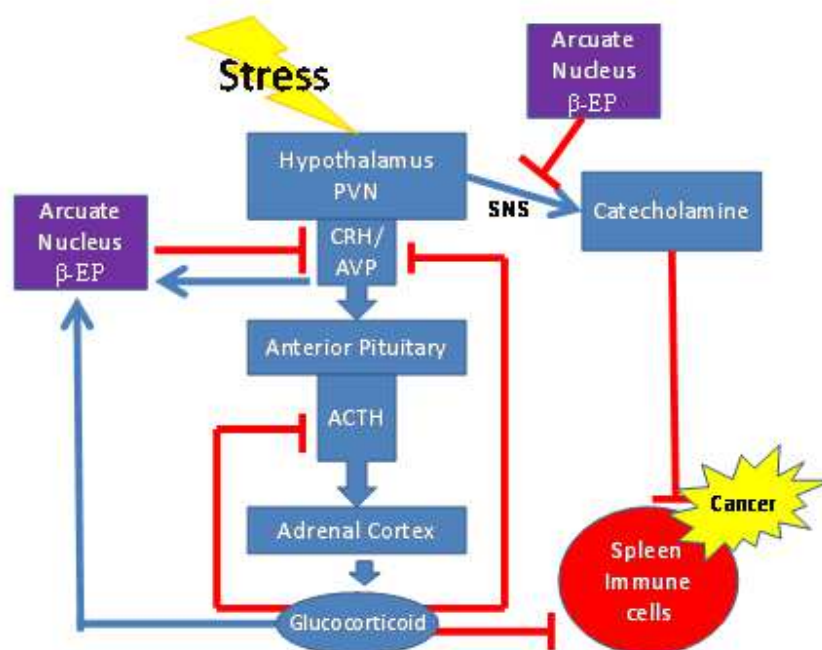
Figure 3:Effect of hypothalamic β-EP on stress and immune activity.

The response of body to stress is tightly regulated. Stress stimulates the release of CRH and AVP from the PVN of the hypothalamus. CRH and AVP get into the pituitary to induce the release of ACTH. ACTH is released into the blood circulation and induces the release of glucocorticoids from the adrenal cortex. Glucocorticoids in turn inhibit the release of CRH/AVP and ACTH, which is negative feedback. Glucocorticoids as well as CRH also induce the release of β-EP from the arcuate nucleus. β-EP is able to inhibit the release of CRH, therefore suppress the activity of the HPA axis. Another pathway that is activated by stress is the SNS, which releases catecholamines into different organs. SNS is also inhibited by β-EP. Both glucocorticoids and catecholamines released during stress inhibit the activity of immune cells in the spleen, which is important for the detection and elimination of abnormally growing cells which may turn into cancer. Since β-EP is able to inhibit both of the stress pathways, it is possible that β-EP may increase immune function and inhibit cancer growth.

In summary, hormones secreted during stress from HPA axis and SNS have inhibitory effects on immune functions against infection and cancer growth. Therefore ways to reduce the detrimental effects caused by a hyperactive stress axis may form a novel method in recovering immunity in a variety of cases.

1.6.2 Stress and cancer

Cancer is a class of diseases characterized by uncontrolled abnormal cell growth, invasion, and metastasis. It is a heterogeneous group of diseases with multiple causes, and immunological involvement varies across different cancers. As early as the ancient time, researchers started to notice a correlation between stress and the occurrence of cancer. About AD 200, Galen wrote that melancholic women were more susceptible to "swelling" of the breast than were sanguine women. Increased incidence of lymphatic, haematological and respiratory cancers was reported in the parents of accident victims and in war-bereaved parents, compared with that in non-bereaved members of the population [255]. On the other hand, behavioral interventions aimed at reducing stress and increasing optimism in cancer patients have been shown to enhance immunity and to reduce tumor growth in mammary and prostate cancer patients [19, 256].

Similar effects of stress on cancer were also observed in animal models. It was reported that chronic high levels of stress can increase carcinogenesis in rats [257]. Chronic behavioral stress also enhances mammary and ovarian carcinogenesis in the rat model [17, 258]. The immune system may be an important link between stress and cancer. The immune system is able to distinguish between "self" and "non-self", and recognize and attack the abnormally transformed tumor cells that lost MHC I protein. Therefore stress, which suppresses the ability of immune function to kill tumor cells, may lead to a higher susceptibility to cancer in humans and animals. In both control and fetal alcohol exposed animals which have hyper-active stress systems, stress was found to decrease the numbers of lymph node pan T and $CD4^+$ T cells, and decrease numbers of total peripheral blood lymphocytes in male rats [95]. Rats unable to escape from electric shock had earlier tumor appearance, enlarged tumors, impaired tumor rejection, reduced lymphoproliferative response, and decreased survival time compared with those given the opportunity to escape the shock [259-260].

Another mechanism by which stress enhances tumor growth is the disturbance of the functionality of the body to recognize and repair errors in DNA replication. Stress has been associated with low concentrations of O6-methyl-transferase, an important DNA repair enzyme induced in response to carcinogen damage, as shown in the splenic lymphocytes of rats subjected to rotational stress [261]. It was also observed that behavioral stress increased frequency of exchanges between sister chromatids in rat cells, which reflects the genomic instability that could be a potential cause of cancer [262]. Psychiatric patients were also found to have significant impairment of DNA repair in the blood after x-radiation compared to normal controls [263].

Many hormones produced in reaction to stressors have cancer-promoting effects. In addition to the previously discussed immune-modulatory effects of these hormones, they may directly affect cancer progression. In human studies, CRH peptide has been found in a small percentage of prostate, thyroid, lung, breast, and GI tumors and in higher percentages of ovarian, endometrium, and skin malignancies [264]. CRH receptor expression is also reported in ovarian, endometrial, breast, skin and liver, where they may mediate growth and apoptotic, immune and metastatic parameters [264]. The role of the CRH receptor in the growth of certain human cancers is supported by direct and indirect evidence [265]. Catecholamines produced by the activated SNS were also shown to promote cancer cell growth through binding to β2-adrenergic receptors and activation of cAMP-PKA signaling pathway [258]. Epinephrine reduces sensitivity of cancer cells to apoptosis through interaction with β2-adrenergic receptors and inactivation of the proapoptotic protein BAD by cAMP-dependent protein kinase [266]. In an orthotopic mouse model of breast cancer, stress-induced β-adrenergic signaling was shown to significantly increase metastasis to distant tissues including the lymph node and lung, and this effect could be inhibited by a β-antagonist [267].

NK cells are important components of the innate immune system that forms the first line defense against cancer development [16]. Low NK cell activity is consistently observed in depressed

adults and children [268]. Swimming stress increased the mortality and metastatic development of two NK-sensitive tumor cell lines but not the metastasis of an NK-insensitive tumor cell line [269]. Both swimming stress and abdominal surgery stresses suppressed NK activity for a duration that parallels the effect of stress in increasing tumor metastasis, indicating that stress-induced NK suppression plays a role in enhancing tumor metastasis [269]. Not only physical stressors, but psychological stressors can also increase tumor development in experimental animals. As group animals, social isolation is a stressor for rodents and creates similar effects as seen in humans who feel isolated. Experiments with rodents have shown that social stressors decrease NK cell activity and enhance the metastasis of transplantable tumors [270-271]. Isolation stress could affect various steps during tumor metastasis, including the direct stimulation of tumor growth at metastatic sites and the stimulation of angiogenesis through HPA activity [272] and through the suppression of cell immunity. In conclusion, animal studies show that stress conditions might promote the initiation and progression of cancer by impairment of immune functions that are relevant to immune surveillance, mainly NK cell activities.

Stress increases the expression of IL-1, IL-6, and TNF-α production from macrophages and monocytes. Most organ-related carcinomas are associated with high concentrations of TNF-α, which inhibits the activity of tyrosine phosphatase. Tyrosine phosphatase in turn diminishes expression of MHC-I antigen on the cell surface. Stress is also known to inhibit expression of MHC-II. With decreased expression of MHC-I and –II on the cell surface, stress permits the abnormal cells to escape immune surveillance. Stress also increases expression of vascular endothelial growth factor (VEGF), matrix metalloproteinase 2 (MMP2) and MMP9, as well as angiogenesis, which promotes tumor growth in a mouse model of ovarian carcinoma [258].

1.7 Use of nanosphere and cAMP in central nervous system

Nanotechnology refers to the particular technological method of precisely manipulating atoms and molecules for fabrication of macroscale products. The term nanoparticle describes matters with one dimension sized from 1 to 100 nanometers, as defined by the National Nanotechnology Initiative in the US. The range can be extended to 1000 nanometers in some cases. This range is about the same scale as the sizes of virus (45~130 nm), ribosome (30 nm), antibodies (12 nm) or hemoglobin (6.5 nm). Nanoparticles readily form suspension in different solvents because the interaction of the particle surface with the solvent is strong enough to overcome density differences. Nanoparticles have a high surface area to volume ratio.

Nanoparticles composed of different materials have been formed, including metal (gold, silver, palladium, and platinum), oxides (alumina, titania, and silica), glass, ceramic, organic polymers (polystyrene, melamine, polylactide), dielectric, semiconductor, and hybrid structures. They are widely used in biomedical applications as drug carriers or imaging agents and are under extensive research. Semi-solid nanoparticles like liposomes are also used as delivery systems for anticancer drugs and vaccines.

There are five major categories of nanoparticle systems for drug delivery: dendrimers, liposomes, micelles, polymeric nanoparticles and nanocrystals [273]. Polymer-based drug delivery systems are the focus of intense clinical and scientific interest because of their stability and ability to encapsulate and protect substances as well as to introduce specific functionality via surface modification. The polymer-based system delivers drugs in a highly localized manner. By using this method, drug delivery can be concentrated at a specific anatomic site or tumor, while minimizing drug exposure and side effects at sites where the drug is not needed. Currently, there are several methods of attaching biological ligands to polymer particles, including absorption to plain particles, covalent attachment to surface functionalized groups, and attachment of the ligand

of interest to particles that are pre-coated with a binding protein such as streptavidin .
Nanoparticles can be designed to release their cargo in accordance with a change in pH or
oxidative stress [273]. Therefore they have significant clinical interest as they offer the
opportunity to link drug delivery to a specific location or disease state.

Melamine resin is a newly developed polymer nanosphere/microsphere system and is
manufactured by acid-catalyzed hydrothermal polycondensation of methylol melamine in the
temperature range of 70-100 °C without any surfactants. By adjusting the pH value, the
concentration of methylol melamine, and the reaction temperature, monodispersed particles with
a predictable size between 0.1-15 mm can be produced in a one-pot synthesis. Unmodified
melamine resin particles have a hydrophilic, charged surface due to the high density of polar
triazine-amino and -imino groups. The surface functional groups (methylol groups, amino groups,
etc.) allow covalent attachment of other ligands. For special applications, the particles can be
modified by incorporation of other functionalities such as carboxyl groups.

Nanoparticles are extensively studied as drug delivery systems for the CNS. The primary focus is
to cross the blood brain barrier (BBB). The BBB is formed by tight junctions at the level of
endothelial cells of the cerebral capillaries and comprises the major obstacle for transportation of
drugs from the blood to the brain [274]. It was shown that nanotech products, in particular
properly functionalized nanoparticles, spherical particles of approximately 200 nm in diameter,
are able to cross the BBB after intravenous administration and carry the drug to the CNS [275-
276]. The ability of nanoparticles to transport drugs through the BBB may be mediated by
increased retention of nanoparticles in the capillary walls that creates higher concentration
gradient which enhances the transport [275]. They can also gain access in the brain via receptor
mediated endocytosis, transcytosis, inhibiting p-glycoprotein efflux pump, and membrane
permeabilization effects [277]. Nanoparticles can also be used in delivery of poorly water-soluble
drugs [278] or in target-specific delivery of drugs through systematical injection. For example,

tumor-specific antibodies can be bond to nanospheres that contain anti-cancer drugs, therefore guiding the nanospheres to bind to specific tumor cells and release the drugs at the site of tumor [279].

Cyclic adenosine monophosphate (cAMP) is an important second messenger inside the cells. It relays the signal from water-soluble hormones, which cannot pass through the cell membrane, into the cells, and activates the protein kinase A (PKA) pathway, finally leading to changes in gene transcription or activation of ion channels. PKA signaling is a critical regulator for neuronal or gilal differentiation in the developing brain and several neuronal cell lines. It has been reported that cAMP and its analogs enhance regeneration of the adult mammalian central nervous system. During the process of CNS injury and repair, there are components of myelin that block axon growth and neuronal regeneration [280-281]. However, these obstacles can be overcome by dibutyryl cAMP (dbcAMP), a membrane-permeable cAMP analog through a PKA-dependent mechanism, resulting in increased axon extension and neuronal regeneration [282-284]. The action of dbcAMP may also involve alterations in neuronal gene expression through activation of the cAMP element-binding protein or mitogen-activated protein kinase signaling [285].

Neuronal stem cells are multipotent cells that have the property of self renewal [286]. They can be isolated from various parts of the brain and differentiated into neurons, astrocytes and oligodendrocytes in the presence of various neurotrophic factors [287]. Many studies have used dbcAMP to induce the differentiation of different types of neuronal stem/progenitor cells to certain phenotypes [282, 288-291]. For example, treating the hippocampal progenitor cell line HiB5 with dbcAMP caused dramatic morphological changes including neurite outgrowth, increased expression of neuronal markers, and decreased expression of nestin (a marker for neural precursor cells) and glial fibrillary acidic protein (GFAP, a marker for astroglia) [282]. Also, dbcAMP could induce the differentiation of the immature human neuroblastoma cell line NB69 into dopamine or acetylcholine neurons [289]. In fetal rat midbrain neuronal cultures, dbcAMP

increases the levels of dopamine and number of tyrosine hydroxylase (TH)-immunoreactive neurons [289]. dbcAMP also promoted the differentiation and reduced migration of cortical astroglia after transplantation, keeping them in the site of injection [292].

However, the cell type achieved by dbcAMP-induced differentiation seems to be largely dependent on the specific region of tissue where the stem/progenitor cells derive from. Our lab has reported a method to differentiate β-EP neuronal precursor cells from hypothalamic NSCs by using dbcAMP, and pituitary adenylate cyclase-activating peptide (PACAP) as inductive signals [221]. When transplanted into PVN in the hypothalamus, these *in vitro*-differentiated β-EP neurons can be integrated into this site, and produce β-EP peptide hormone. Increased β-EP peptide level can be detected in hypothalamus, but not in blood plasma. Since β-EP plays an important role in regulation of stress and immune system, the method to increase β-EP level in the hypothalamus may have great significance. We have shown that transplantation of β-EP neurons into hypothalamus could reduce the response of CRH to a lipopolysaccharide (LPS) challenge, and significantly increase NK cell cytolytic activity in rats [221]. It has been shown that these β-EP transplants can inhibit prostate tumor development in a carcinogen-induced rat prostate cancer model [221], possibly by increasing NK cell activity, reducing the body's inflammatory milieu, and some other mechanisms. Out lab has also shown that *in vitro*-produced β-EP neuronal transplants can eliminate NK cell functional deficiency and recover the decrease of IFN-γ in fetal alcohol exposed rats [113].

Although the β-EP neuronal transplants have significant benefit on immune functions against different diseases, it has drawbacks that make this technique hard to be adapted to clinical application. The major problem is to obtain stem cells from fetal origin. Another problem might be transplant rejection caused by host versus graft response, although our study in rats suggests that NSC-derived β-EP cells are not rejected in host animals in various strains, which may be due to the lack of MHC-I protein in NSC. One possible solution to this problem is to differentiate β-

EP neurons from NSCs of the host itself. The main sources of NSCs in the adult mammalian CNS are the subventricular zone (SVZ), the external germinal layer of the cerebellum, the subgranular zone of the dentate gyrus, and the ependymal layer of the spinal cord [293-294]. Since neuronal differentiation from NSC persists in the adult, and most of the neurons in the hypothalamus are derived from the proliferative neuroepithelium of the third ventricle [293], it is possible to induce NSC to differentiate into β-EP neurons *in vivo* by administering cAMP-activating agents into the third ventricle. To immobilize and ensure a slow release of cAMP and PACAP molecules, nanosphere/nanoparticles can be used as carriers.

The combination use of dbcAMP and a microsphere/nanosphere delivery system has been investigated. The delivery system of dbcAMP using poly(lactic-co-glycolic acid) (PLGA) hydrogel was first described in 2009, showing abilities to stabilize the drug and control the speed of drug release [295]. In cell replacement therapy for injury of the CNS, such as the spinal cord, these dbcAMP-containing microspheres were used in order to induce differentiation of NSCs to functional neurons. Under normal conditions, NSCs transplanted into the spinal cord have a greater tendency to differentiate into glia cells instead of neurons *in vivo* [296-297]. Since dbcAMP was shown to promote differentiation of NSCs to neurons, studies were conducted using dbcAMP to promote NSCs differentiation in the site of spinal cord injury [288]. In this study, dbcAMP was encapsulated in PLGA polymer microspheres in order to achieve location- and time-controlled release. These dbcAMP-delivering particles were transplanted together with NSCs to the site of spinal injury, and significantly increased the NSCs survival and number of NSCs differentiated into neurons [288]. Encapsulation of dbcAMP in PLGA microspheres lead to prolonged release and continued functionality *in vitro* [298]. This method rescues mesenchymal stem cell-induced inhibition of axonal regeneration, as well as capillary formation, and is beneficial for the functional improvement [298].

In conclusion, nanosphere/microsphere may be a good delivery system of dbcAMP for the survival and differentiation of NSCs at a specific region in the brain and may be used for generation of β-EP neurons from the hypothalamus.

2   Objectives of the Thesis

The overall aim of this thesis work is to identify the role and regulatory pathway of hypothalamic β-EP on the stress axis activity, immune function and mammary cancer development in fetal alcohol exposed rats.

There are three objectives:

1. **Objective 1**: Determine whether activation of β-EP neurons can suppress the stress axis, increase immune function and inhibit mammary cancer

2. **Objective 2**: Determine whether activation of β-EP neurons can reverse the effect of fetal alcohol exposure on the stress axis, immune function and cancer susceptibility

3. **Objective 3**: Determine the pathway through which the β-EP neurons control cancer development

CHAPTER 2

3   **Chapter 2: β-EP neuronal transplantation suppresses the stress axis, increases immune function and inhibits mammary cancer**

3.1   Introduction

Breast cancer is the most frequent malignant disease among women. The National Cancer

Institute estimated that there would be 40,170 deaths due to breast cancer [292], while the

American Cancer Society predicted 192,370 new cases of invasive breast cancer among

American women in the year 2009 [299]. Stress has been shown to be a tumor-promoting factor

[236, 258, 300-302]. Emerging evidence suggests that chronic neurobehavioral stress can

promote various tumor growth and progression secondary to sustained activation of sympathetic

nervous system and inhibition of parasympathetic nervous system [14-15, 303]. Stress can

significantly affect many aspects of the body's immune systems. For example, higher levels of

stress were shown to be associated with decrease in natural killer (NK) cell lysis activity,

macrophage migration activity, decrease of T cell population, decreased lymphocyte proliferation

following infection, and decrease in interferon-γ (IFN-γ) levels (reviewed in ref. [14]). These

factors are reported to be important components of immunity against cancer [15-16]. Therefore,

manipulations to control the body's stress response may be beneficial to increase immunity and

fight against cancer. β-endorphin (β-EP), an endogenous opioid polypeptide primarily produced

by the hypothalamus and pituitary gland, is known to have the ability to inhibit stress hormone

production, produce analgesia and a feeling of wellbeing [18-19]. β-EP is a cleavage product of

proopiomelanocortin (POMC), which is also the precursor hormone for adrenocorticotrophic

hormone (ACTH) and melanocyte-stimulating hormone (MSH). β-EP neuronal cell bodies are

primarily localized in the arcuate nuclei of the hypothalamus, and its terminals are distributed

throughout the CNS, including the paraventricular nucleus (PVN) of the hypothalamus [303]. In

the PVN these neurons innervate corticotropin releasing hormone (CRH) neurons and inhibit

CRH release [222], while a μ-opioid receptor antagonist increases it [225]. During stress, secretion of CRH and catecholamine stimulate secretion of hypothalamic β-EP and other POMC-derived peptides, which in turn inhibit the activity of the stress system [225]. β-EP is known to bind to δ- and μ-opioid receptors and modulate the neurotransmission in sympathetic neurons via neuronal circuitry within the PVN to alter NK cell cytolytic functions in the spleen [113, 223]. Abnormalities in β-EP neuronal function are correlated with a higher incidence of cancers and infections in patients with schizophrenia, depression, and fetal alcohol syndrome and in obese patients [9, 21-22, 24, 226, 304-305].

We have recently shown that the neural stem cell–derived β-EP neurons, when transplanted into the PVN, remained at the site of transplantation, decreased lipopolysaccride (LPS)-induced levels of hypothalamic CRH and plasma corticosterone, increased NK cell cytolytic function and anti-inflammatory cytokines productions in response to immune challenge, and suppressed carcinogen-induced prostate cancer development in rats [113, 221]. It is not known whether β-EP transplants prevent mammary tumor growth. Also, the effects of β-EP neural activation on cancer progression and its metastasis to distant tissues are not evaluated. In this study we examined the effect of transplantation of *in vitro* differentiated β-EP neurons from fetal neuronal stem cells into the hypothalamus on tumor incidence, growth, malignancy rate, and metastasis using a rat model of mammary cancer. Additionally, we determined immunological and neurochemical changes pertinent to β-EP action on tumor.

3.2   Materials and Methods

Animals.

Adult Sprague-Dawley (SD) and Fischer 344 male and female rats were purchased from Charles River (Wilmington, MA) and maintained in a controlled environment with a 12 h light/dark cycle at Bartlett Hall Animal Research Facility of our institute. Male and female rats of each strain

were bred and their fetuses or offspring were used in this study. All the animals were housed individually, allowed free access to regular rat chow, water and maintained their normal physical activities throughout the study. Animal care was performed in accordance with institutional guidelines and complied with National Institutes of Health policy.

Preparation of β-EP cells from neural stem cells.

We isolated neural stem cells from 17 days old fetal rat brains of Sprague Dawley rats and then differentiated these cells into β-EP neurons in culture to use in this study. We used cAMP and pituitary adenylate cyclase-activating polypeptide (PACAP) to differentiate β-EP neurons from rat fetal neural stem cells, as we have recently described [221]. To control for transplantation, we used cortical cells prepared from 17-day-old fetal rat brains. The justification for the use of cortical neurons as control is previously described [221]. Prior to transplantation, differentiated β-EP cells were dissociated and resuspended at a concentration of 20,000 viable cells/µl in HEPES-buffered DMEM-containing serum supplement (SS; 30 nM selenium, 20 nM progesterone, 1 µM iron-free human transferrin, 5 µM insulin, 100 µM putrescine and antibiotics), cAMP (10 µM) and PACAP (10 µM) for the transplantation. Cells were placed on ice throughout the grafting session. Cell viability, assessed by the trypan blue exclusion assay, was routinely greater than 90%. The composition of the differentiated cultures, with respect to the absence of undifferentiated neural stem cells and the presence of mature β-EP -producing cells, was verified before grafting by staining for the immature neural marker nestin and/or vimentin, and for β-EP using immunocytochemistry as described previously [221].

Determining the functionality of β-EP cells.

We verified the functionality of the transplanted cells by doing a physiological test in the transplanted animals followed by confirming the presence of β-EP neurons at the site of transplantation after the termination of the experiments. We have previously shown that animals

transplanted with β-EP neurons have increased expression of POMC mRNA and decreased expression of CRH mRNA in the PVN and reduced response of plasma corticosterone following a LPS challenge [113]. In this study, we verified the function of transplanted β-EP neurons by determining the changes in corticosterone response to LPS. We used a 100 µg/kg dose of LPS for a period of 2 h (which was found to be an effective dose; [110]) to determine the changes in the plasma corticosterone responses. After termination of the experiment, the brain was collected and processed for histochemical verification of the presence of transplanted β-EP cells in the PVN of the hypothalamus using immunohistochemical methods [221]. PVN nuclei do not contain β-EP neuronal cell bodies in situ [222] (control transplanted PVN did not have any β-EP staining; data not shown), hence the immunocytochemically detected β-EP cells in this area were considered transplanted cells. All the animals included in cancer study showed transplanted β-EP cells in the PVN.

Tumor induction and characterization.

In order to determine the effect of β-EP neuronal transplants on mammary tumor growth and progression, 50 days old ovary intact virgin Sprague Dawley rats were injected i.p. with a dose of MNU (50 mg/kg body weight). Six weeks after the MNU injection, animals were anesthetized and injected with cortical neurons (Control) or β-EP neurons in both sides of PVN of the hypothalamus using stereotactic procedures described by us previously [221]. No tumors were detected at this time. Beginning 1 week, when the animals recovered from the brain surgery, animals were weighed and palpated every week to check for tumor growth. Tumor length, width and depth were measured with a calibrator as previously described [9]. Sixteen weeks after the MNU injection, animals were sacrificed, tumors were collected, and slices of tumors were immersed in formalin and processed for histology staining. Fixed tissue was dehydrated, cleared and paraffin infiltrated overnight using a tissue processor. The tissues were paraffin-embedded, sectioned into 5 µm thick slices and placed on slides. One slide from each tissue was stained with

hematoxylin and eosin (H&E) to evaluate tissue histology and tumor pathology. Slides were evaluated by a pathologist blinded to treatment. Ductal/cystic hyperplasia was defined by increased proliferation of benign glandular structures, with predominantly regular cells and nuclei. Adenomas were defined by a more solid phase glandular structure with regular cells and nuclei predominating. Adenocarcinomas presented primarily as solid-phase lesions containing many atypical and anaplastic cells, a high mitotic rate (including numerous atypical mitoses) and observable zones of tumor necrosis (apoptotic) and some show significant invasiveness.

Immunohistochemical localization of various proteins.

Thin paraffin sections (5 μm) of mammary tumors were stained using the ABC Elite Vectastain kit (Vector Labs, Burlingame, CA) according to manufacturer's instructions using various primary antibodies. Primary antibodies for immunohistochemistry were used as follows: polyclonal rabbit antibodies against TNF-α (1:250), NF-kB (1:100), E-cadherin (1:250) and N-cadherin (1:250) (all from Abcam, Cambridge, MA), as well as Snail (1:200), Slug (1:200), and Twist (all from Santa Cruz Biotechnology, Santa Cruz, CA). After the primary antibody incubation and PBS wash, sections were incubated with peroxidase-coupled anti-rabbit Ig ImmPRESS reagent (Vector Laboratories, Inc., Burlingame, CA). Antigen localization was achieved by using the 3,3′-diaminobenzidine-peroxidase reaction and sections were dehydrated, and coverslipped. To evaluate the immunohistochemical staining, sections were photographed using Nikon-TE 2000 inverted microscope. Intensity of staining was categorized as negative (-) and strongly positive (+++).

Western blotting.

For Western blotting, tumor tissue extracts equivalent to 50 μg total protein were separated by 4-20% SDS/PAGE and transferred overnight to immobilon-P PVDF membranes. Membranes were incubated with primary antibody for 18 h at 4°C in blocking buffer. Membranes were then

washed and incubated with peroxidase conjugated secondary antibody (1: 5000) for 1 h.

Afterwards membranes were washed and then incubated with ECL Western blot

chemiluminescence reagent (Pierce, IL, U.S.A.). Membranes were exposed to X-ray films and

developed using X-Ray developer. Actin served as an internal control for loading and transfer of

equal amounts protein samples. Details of all primary antibodies used are described above.

Metastasis study.

We used the MADB106 cells, a Fischer 344 rat mammary adenocarcinoma cell line maintained in

DMEM containing 10% FCS. Female Fischer-344 rats were transplanted with β-EP cell

transplants or control cell transplants at 50 days of age. After about 2 weeks, these PVN cell

transplanted rats were inoculated with MADB106 tumor cells (100,000 cells/0.2 ml/rat) into the

jugular vein under sodium Nembutal solution (50 mg/kg) anesthesia. Wounds were closed with a

surgical clip, and rats were left on heating pad till recover. Rats were sacrificed at 4 weeks, after

the tumor cells inoculation, whole body of each animal was inspected for the presence of visible

tumors. Tumors were only located in lungs in most animals, and at the inoculation site in a few

animals. Lungs were collected, fixed in formalin, embedded with paraffin, and sectioned for H&E

staining. Stained slides were examined under the microscope for the verification of the presence

of tumor. Brain tissues were processed for verification of the site and viability of β-EP cell

transplantation.

Determination of pharmacological modification of autonomic influence on immune system on

lung metastasis.

We hypothesize that the beneficial effect of β-EP neuron transplants on immune function is

regulated through autonomic nervous system, and that central β-EP modulate peripheral immune

function by inhibiting the sympathetic activity and activating the parasympathetic activity.

Metaproterenol (MET) is an agonist for β-receptors, which are a class of G protein-coupled

receptors that are targets of the catecholamines, especially norepinephrine and epinephrine [241, 306]. Methyllycaconitine (MLA) is a selective antagonist of α7 nicotine acetylcholine receptor (α7 nAChR) [307]. By injecting MET and MLA i.p., we mimicked the stimulation of sympathetic nerves and inhibition of parasympathetic nerves. We also used naloxone, an opiate receptor antagonist to test whether β-EP neuronal effects is acted via opiodergic receptor [223]. Forty-eight β-EP neuron transplanted Fischer rats and 48 control rats were divided into 8 groups and treated with saline, naloxone (10 mg/kg body weight), metaproterenol (MET; 0.8 mg/kg body weight), or methyllycaconitine (MLA; 2.5 mg/kg body weight) for 8 days (the doses used for these drugs are recognized as effective biological doses). A day after the first injection of these agents, animals were inoculated with MADB106 cells (100,000/0.2 ml/rat). Twenty-four hours after tumor cell inoculation, blood was collected by orbital puncture. Plasma was collected, and PBMCs were separated for migration assay and NK cytolytic assay. These animals continued receiving the blocker treatments for 7 days. After 4 weeks they were sacrificed, their lungs were fixed in formalin and processed to H&E staining for determination of cancer pathology. Brain tissues were processed for verification of the site and viability of β-EP cell transplantation.

Immune reaction after tumor cell Inoculation.

We checked immune reaction to tumor cell inoculation in animals after they received β-EP neuron transplants or cortical neuron transplants for 4 weeks. Animals were anesthetized with Nembutal, 1 ml of blood was drawn from jugular vein, and MADB106 cells (100,000/0.2 ml/rat) were inoculated into the jugular vein. After 24 hours, animals were anesthetized again. 1 ml of blood was drawn from the jugular vein, and then animals were sacrificed by decapitation. Vein blood before and after tumor inoculation was used for flow cytometry to determine cell populations in PBMC as described previously [241]. Trunk blood peripheral blood mononuclear cells (PBMCs) and splenocytes were used for NK cell cytolytic assay [221] and macrophage migration assay (CytoSelect 96-well cell migration assay, 5 μm, Cell BioLab). Trunk plasma was

used for multi-cytokine assay. After termination of experiment, spleen were obtained and splenocytes were prepared and used for measuring mRNA levels of various cytokines and cytotoxic factors using real-time RT PCR methods as described by us previously [241].

Plasma analysis of hormones and cytokines.

Plasma was analyzed for corticosterone levels by a competitive enzyme-linked immunosorbent assay (ELISA) (Immunodiagnostic Systems, Fountain Hills, AZ) according to manufacturer's recommendations. All samples were run on one 96-well plate for each variable. Plasma cytokine levels were measured by AssayGate, Inc (Ijmasville, MD) using their multiplex platform which measures multiple cytokines in a single plasma sample. Each sample was measured in triplicate. Immune related cytokines data are presented in figures.

Statistics.

Differences in average tumor incidence, tumor number and tumor volume were assessed using two-way ANOVA with a Bonferroni post-test at the level of $\alpha = 0.05$. To evaluate tumor type, a Chi-square test was performed. Differences in tumor incidence and immune after various drugs were assessed using one way ANOVA with a Newman-Keuls post-hoc analysis at the level of $\alpha = 0.05$. T-tests were used to evaluate the differences in various protein and cytokine levels.

3.3   Results

β-EP neuronal transplantation into the hypothalamus suppresses mammary tumor growth and progression.

We used the *in vitro* produced β-EP neuron in this study (Figure. 4A). Prior to testing β-EP neuronal effects on mammary cancer growth, we evaluated the functionality of β-EP-neuron transplantation into the hypothalamus following long-term transplants. For this, female rats at age of 50 days were implanted with β-EP neuronal cells or control cells in both sides of PVN. After

two to three months of cell transplants, these rats were inspected for changes in reproductive cyclicity and body growth. Like the untreated rats, both control and β-EP cell transplanted rats showed regular 4-5 day estrous cycle. Body weights of untreated control, control cell transplanted rats and β-EP cell transplanted rats were similar (Untreated, 243 ± 6; control cell transplanted, 251 ± 5; β-EP cell transplanted, 253 ± 6; N = 8-10), After 6 months, they were used to determine their plasma corticosterone response to LPS, as a functional test of β-EP neuronal activity [113], and their brains were employed for immunohistochemical verification of transplanted neurons in the PVN. Transplanted β-EP cells remained at the site of transplantation, as they were detected in the PVN by immunostaining (Fig.4A & B). As reported by us previously [113], β-EP transplanted (β-EP) animals showed lower plasma corticosterone response to LPS (Fig. 4C).

We used N-methyl-N-nitrosourea (MNU) to induce mammary cancer in rats [9]. Six weeks after the administration of MNU, animals were anesthetized and injected with *in vitro*-differentiated β-EP neurons or *in situ* cortical neurons, which served as the controls, in both sides of the PVN of the hypothalamus [221]. Weekly body weight gains were similar between rats with control transplant or β-EP transplants (Fig. 4D). Weekly measurement of tumor number, length and width for a period of sixteen weeks revealed that β-EP neurons implanted animals had lower tumor incidence, tumor number and tumor volume (Fig. 4E-G). At the termination of the experiment, whole body inspection revealed that tumors were localized only in mammary glands. Tumors from the study were classified by histological analysis (Fig. 4H-L). Histopathological evaluation of tumors showed that, unlike control animals, which had mostly adenonocarinoma (both invasive and non-invasive), most of the β-EP -treated animals had benign adenoma with glandular hypertrophy.
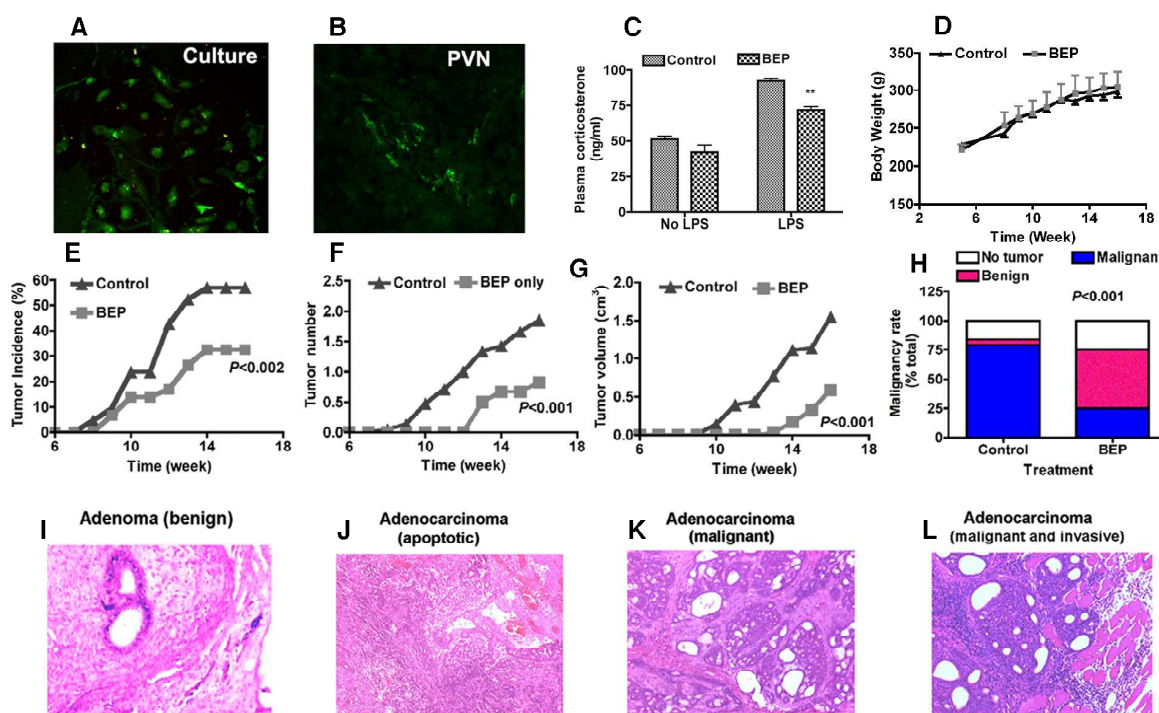
Figure 4: β-EP neuronal transplant in the hypothalamus suppresses mammary tumor growth and progression.

Animals were administered with a single I.P. injection of MNU at 50 mg/kg at 50 days of age. After six weeks of MNU, rats were administered with 20,000 cells/μl of β-EP cells or cortical cells (control) into the PVN bilaterally. Rats were palpated for tumors once a week following injections. **A**. A representative photomicrograph showing β-EP cells in primary cultures after stained for the β-EP peptide by immunofluorescence technique. **B**. A representative immunofluorescence pictures of β-EP cells in the PVN area of the hypothalamus after 6 months of transplants. **C**. Corticosterone response to LPS. **D.** Body weight changes. E. Graph represents percent (tumor incidence) of rats presenting with tumors each week post-injection. **F**. Graph represents average number of tumors per treatment group each week post injection. **G**. Average volume of tumor per animal in each group. **H**. Tumors malignancy rate as determined by histological evaluation. Percentage of each histological tumor type that developed per treatment group. Data of panel C were analyzed using one-way analysis of variance followed by Newman-Keuls post-hoc analysis. Data in panels **D-G** were analyzed using a two-way ANOVA and only the data shown in E-G were found to have significant treatment and time interactions at $P<0.001$ ($n = 12$/group), and panel **F** was analyzed using a Chi-square test with a $P<0.0001$. **G-J**. Representative images of different histological tumor types developed in our model.

Numerous studies have demonstrated that the inflammatory tumor microenvironment potentiates not only tumor development, but also the progression of adenoma to carcinoma and induction, stabilization of epithelial-mesenchymal transition (EMT) in tumor tissues [308-310]. Loss of E-

cadherin and aberrant N-cadherin expression associates with the acquisition of invasiveness and more advanced tumor stage for many cancers including breast cancer [311]. Therefore, we determined the expression of pro-inflammatory cytokine TNF-α and pro-inflammatory NFkB, transcription factors linked to the morphogenetic processes causing EMT (Snail, Slug and Twist) expression, and expression of mesenchymal adhesion factor (N-cadherin) and epithelial adhesion factor (E-cadherin). Immunohistochemical and Western blot determination of various cytokines in tumor tissues obtained from rats with β-EP cell transplants showed reduced levels of TNF-α and NF-kB, as well as Snail, Slug and Twist with concomitant decreased in the level of N-cadherin, and increased the level of E-cadherin in mammary gland as compared to control transplanted animals (Fig. 5A-G). In summary, the cellular and morphological data of tumors suggest the possibility that β-EP neuron transplants have prevented the development of advanced stage carcinoma possibly by suppressing the inflammatory response and repressing EMT factors in tumor tissues.
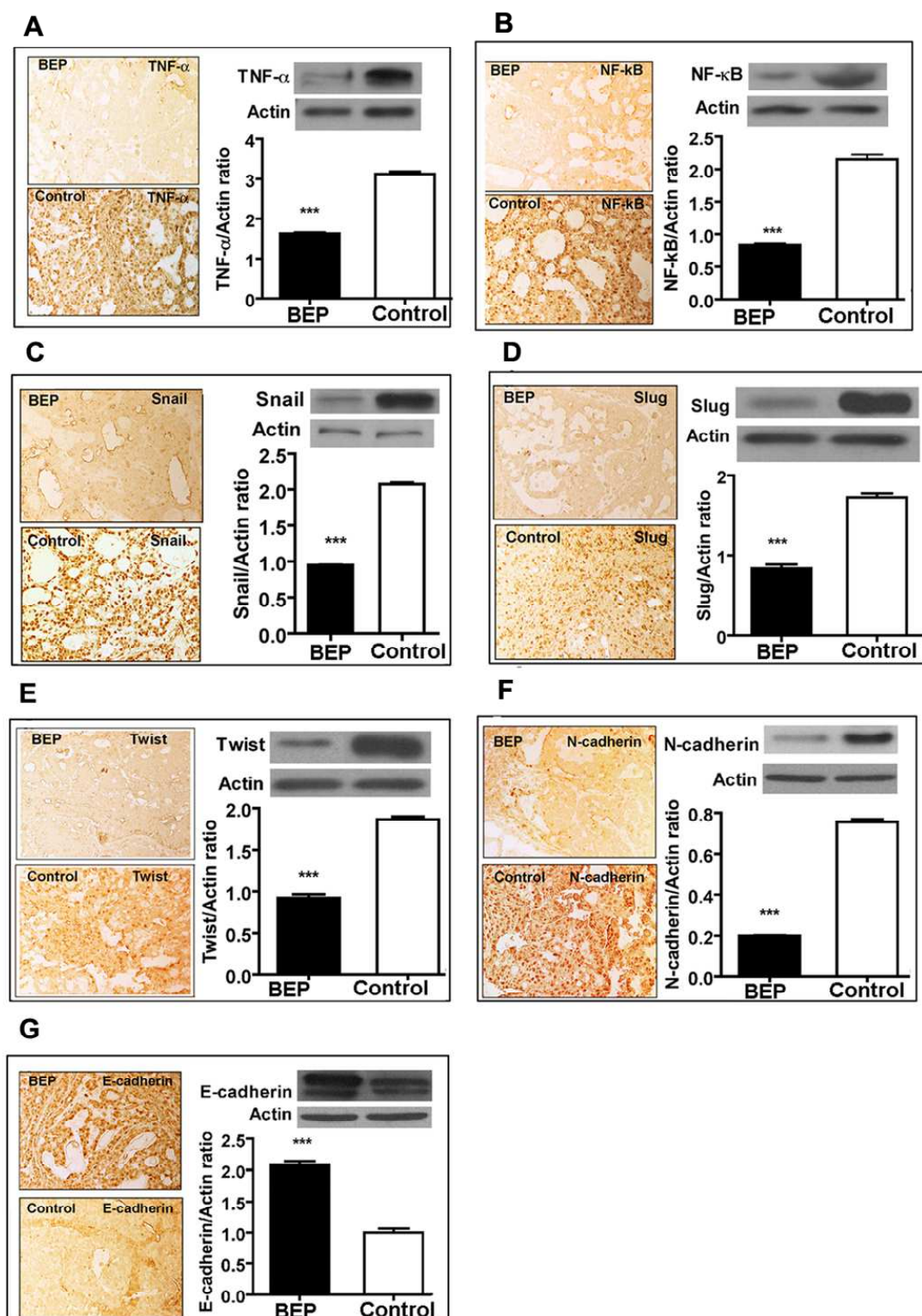
Figure 5: Effects of β-EP neuron transplants on the expression of inflammatory markers (TNF-a and NF-kB), epithelial-mesenchymal transition factors (Snail, Slug, Twist), mesenchymal marker (N-cadherin), and epithelial marker (E-cadherin) in tumor tissues.

Cortical cells were used as control transplants. Tumors were removed from animals treated with MNU followed by β-EP neuron or control neuron transplants as described in Figure 1 legends. Cellular levels of inflammatory and EMT factors were determined by immunohistochemistry (shown on the left of each panel) and Western blotting (shown on the right in each panel). **A**. TNF-a expression. **B**. NF-kB expression. **C**. Snail expression. **D**. Slug expression. **E**. Twist expression. **F**. N-cadherin expression. **(G)** E-cadherin expression. Actin expression served as an internal control. n = 5 rats. ***, P < 0.001 vs. control.

β-EP neuron transplantation into the hypothalamus prevents mammary tumor metastasis to lung.

 In order to determine the effects of β-EP neuron transplants on tumor metastasis, we used non-immunogenic syngeneic MADB106 mammary cancer cells, which are widely used for lung metastasis studies in rats [312]. Four weeks after inoculation of MADB106 cells through jugular vein, 70~80% of inoculated control cell-transplanted animals showed visible multiple tumor foci in lungs (Fig. 6A). Some of these animals also had a single visible tumor at the site where the tumor cells were inoculated (data not shown). None of the β-EP -transplanted animals showed visible tumors either in the lung or any other body sites. Histological examinations of lung tissues identified focal and invasive tumors in control-transplanted rats while no tumors in β-EP transplanted rats (Fig. 6C). These data suggest that the β-EP transplantation completely eliminated retention of MADB106 tumor cells in the lungs.

A Control cell transplanted

B BEP cell transplanted

C BEP cell transplanted / Control cell transplanted / Control cell transplanted

Normal Focal tumor Tumor invasion
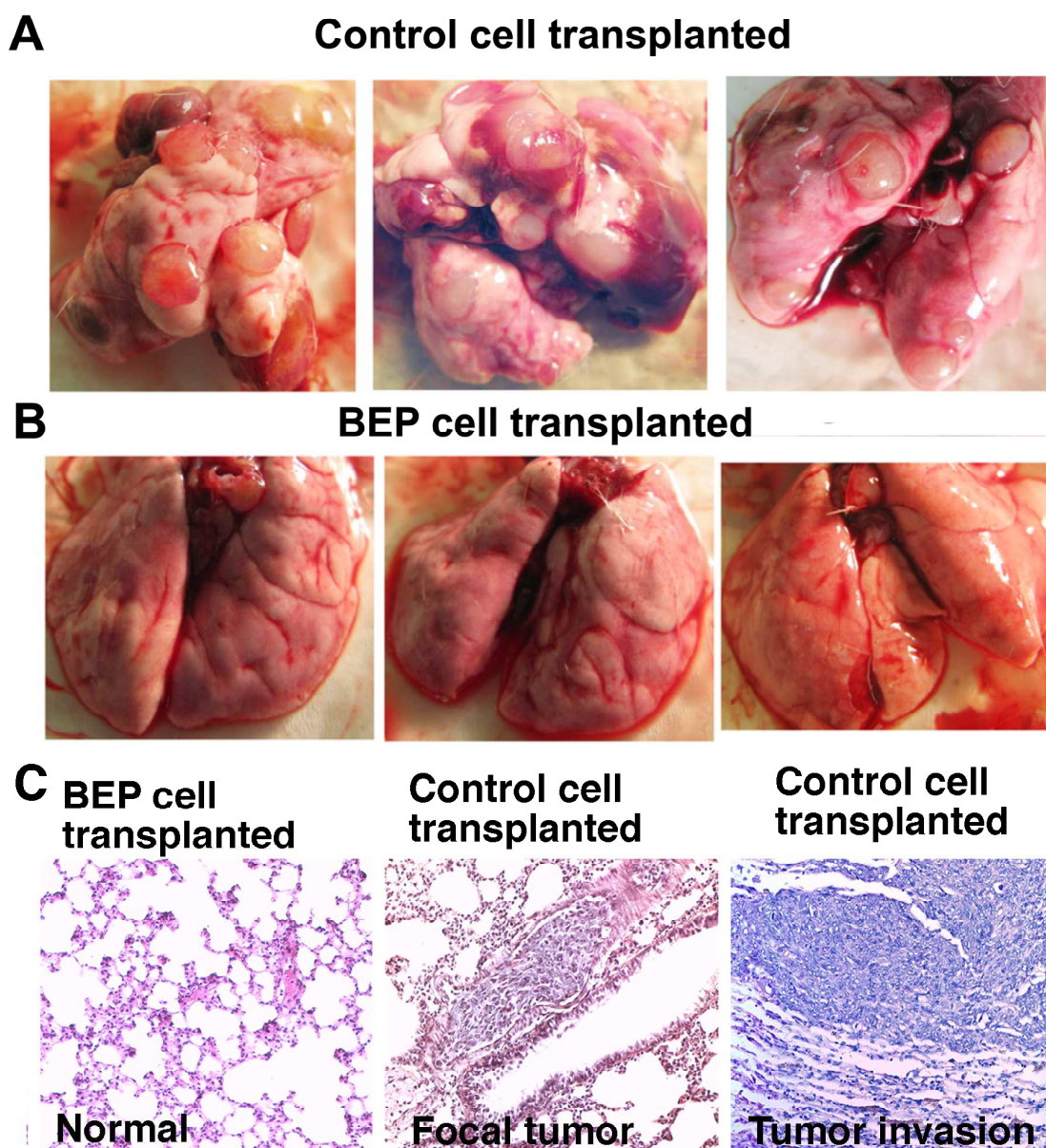
Figure 6: Evaluation of effects of β-EP-cell transplantation on mammary cancer metastasis.

Rats were transplanted with 20,000 viable cells/μl of β-EP cells or cortical cells (control) into two PVN, and then after 4 week they were inoculated with 100,000/0.2 ml/rat of MADB106 cells via jugular vein. **A**. At sacrifice, 70~80% of inoculated animals showed tumor at the position of lung in control but the β-EP inoculated animals did not (representative lung figures). Showing multiple tumor foci in lungs of control cell transplanted rats. **B**. Showing absence of tumor foci in lungs of β-EP cell transplanted rats. **C**. Representative photomicrographs of H&E stained normal and metastatic lung tumor.

Activation of hypothalamic β-EP neurons increases innate immune functions.

Since β-EP transplantation increases immune activity in normal animals and since the innate immune system is critical for tumor cell clearance (reviewed in ref. [313-314]), we hypothesize that the elimination of tumor cells from lungs of β-EP transplanted animals may have been caused by increased innate immune function. Using the MADB106 cancer model, we checked immune response [315] 24 hours following tumor cell inoculation. Several studies have demonstrated that MADB 106 tumor cells metastasize only to the lungs and are eliminated by NK cell cytotoxic activity when injected intravenously [312]. In this study, we have not quantified the cytokine profile for longer observation, because it may be possible that there may be some changes in the cytokines profile by adaptive antitumor immune response only in control cell transplanted rats that may not reflect the innate immune responses as like the early stage of tumor development.

We found that the β-EP -transplanted rats had greater NK cell cytolytic activity in PBMCs and in splenocytes as well as greater macrophage migration and cell proliferation activity than those in control cell–transplanted rats (Fig. 7A). Consistent with these data, we found that splenocytes of β-EP-transplanted rats had higher mRNA and protein levels of NK cell cytolytic activity regulatory factors (e.g., granzyme B; [316]), NK cell activator receptor (e.g., NKG2; [317]), cytokines that is produced after NK activation (e.g., IFN-γ but lower TNF-α; [318]), a chemokine that recruits and activates macrophages (MCP1; [319]), and a protein that is produced in an increased amount from activated macrophages (IL-1b; [320]) (Fig. 7B). Furthermore, measurement of cytokines and protein levels in blood plasma of tumor-inoculated rats showed that the levels of macrophage-regulatory/derived factors (GM-CSF, MIP-1α, IL-18; [321-323]) and an NK cell–regulatory/derived factor (IFN-γ; [318]) are higher in β-EP-transplanted rats than those in control-transplanted rats (Fig. 7C). Also, lower levels of several inflammatory cytokines (IL-1α, IL-12, TNF-α; [324]) were observed in plasma of β-EP-transplanted rats as compared to control-transplanted rats. In addition, β-EP transplants increased NK and macrophage cell

numbers in PBMCs, but decreased their numbers in splenocytes after tumor cell inoculations (Fig. 7D,E), suggesting that the transplants also promoted migration of these immune cells out of the spleen to the blood and/or inhibited emigration from the blood into the spleen to promote defense against tumor cells. These data suggest that the β-EP neuronal supplement promotes innate immunity and produces an anti-inflammatory environment in recipient animals.
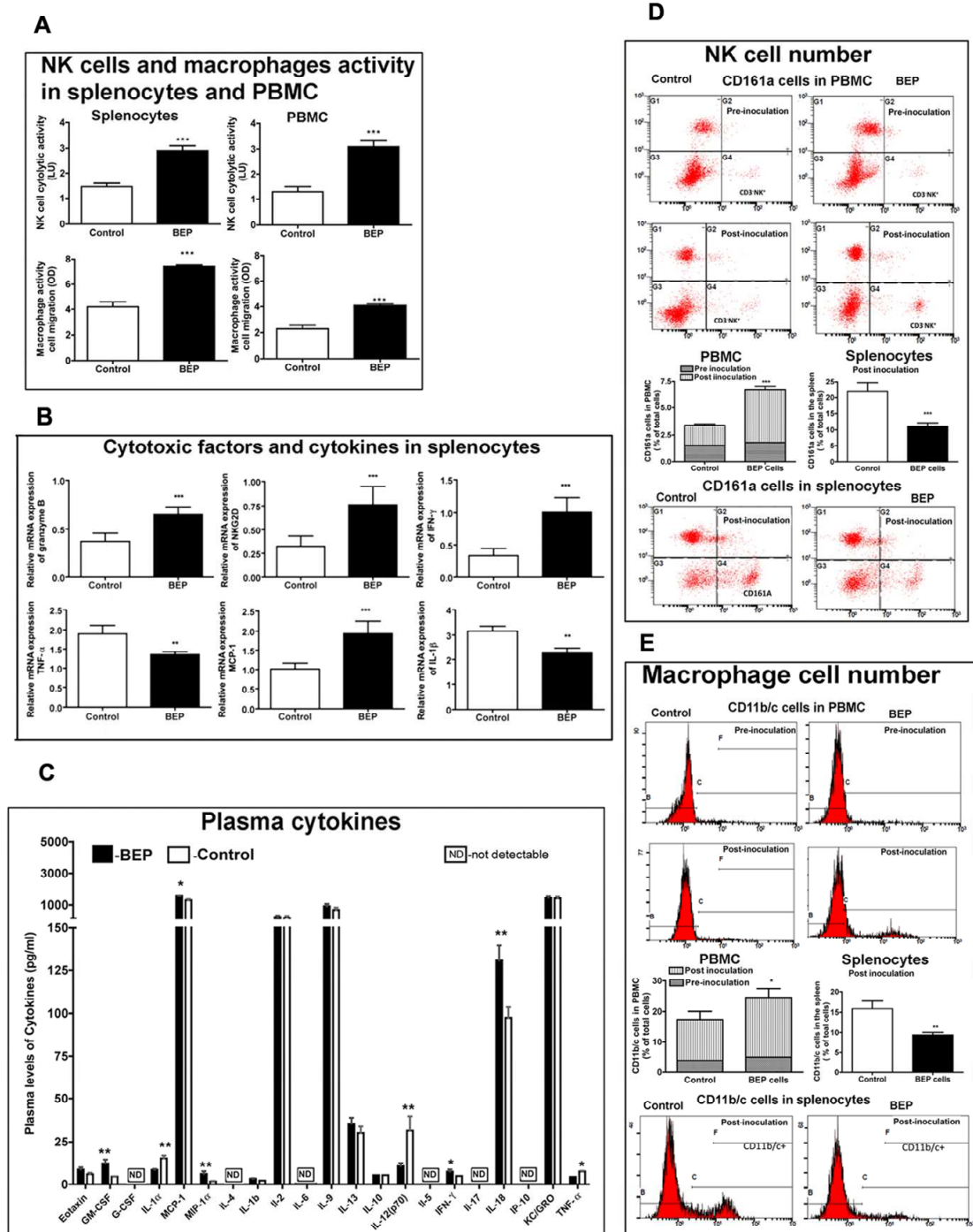
Figure 7: Evaluation of effects of β-EP-cell transplantation on cancer cell retention and immune functions.

Rats were transplanted with 20,000 viable cells/µl of β-EP cells or cortical cells (control) into two PVN, and then after 4 week they were inoculated with 100,000/0.2 ml/rat of MADB106 cells via jugular vein. **A**.

NK cell cytolytic activity and macrophage migration activity in splenocytes or in PBMC at 24 hours after tumor inoculation. **B**. Splenocytes mRNA levels of cytotoxic factors and cytokines genes known to regulate NK cell and macrophage functions at 24 hours after tumor inoculation. **C**. Plasma levels of various cytokines at 24 hours after tumor inoculation. **D**. NK cell populations in PBMC before and after tumor inoculation, and in splenocytes 24h after tumor inoculation. **E**. Macrophage cell populations in PBMC before and after tumor inoculation, and in splenocytes 24 hours after tumor inoculation. n = 8-12 rats. [*], P < 0.05; [**], P < 0.01; [***], P < 0.001 vs. control.

Antagonists of opiate receptor and α7 nicotine acetylcholine receptors and agonists of β-adrenergic receptors prevent β-EP neuronal ability to enhance immunity and suppress tumor metastasis.

β-EP neurons produce opioid peptides and other products of POMC [325]. To address the question whether β-EP alone and/or other peptide products from the transplanted neurons are responsible for the observed actions on the immune system and cancer, we tested the ability of a general opiate antagonist naloxone to block the effects of β-EP neuron transplants on immune activation and metastasis prevention. Additionally, we tested the effects of norepinephrine agonist MET and a α7 nAChR antagonist, MLA. We found that 24 h after tumor inoculation, PBMC NK cell activity (Fig. 8A) and macrophage migration activity (Fig. 8B) were higher in saline-treated β-EP -transplanted animals than in saline-treated cortical cells-transplanted controls. Naloxone, MET, and MLA all had moderate or strong inhibitory effect on basal and β-EP-stimulated NK cell activity and macrophage migration activity. Consistent with these findings, we observed that naloxone, MET, and MLA prevented, at various degrees, the beneficial effect of β-EP in eliminating tumor cell lung retention (Fig. 8C). These data suggest that β-EP neurons activate innate immunity for cancer cell clearance via altering the function of the autonomic nervous system.
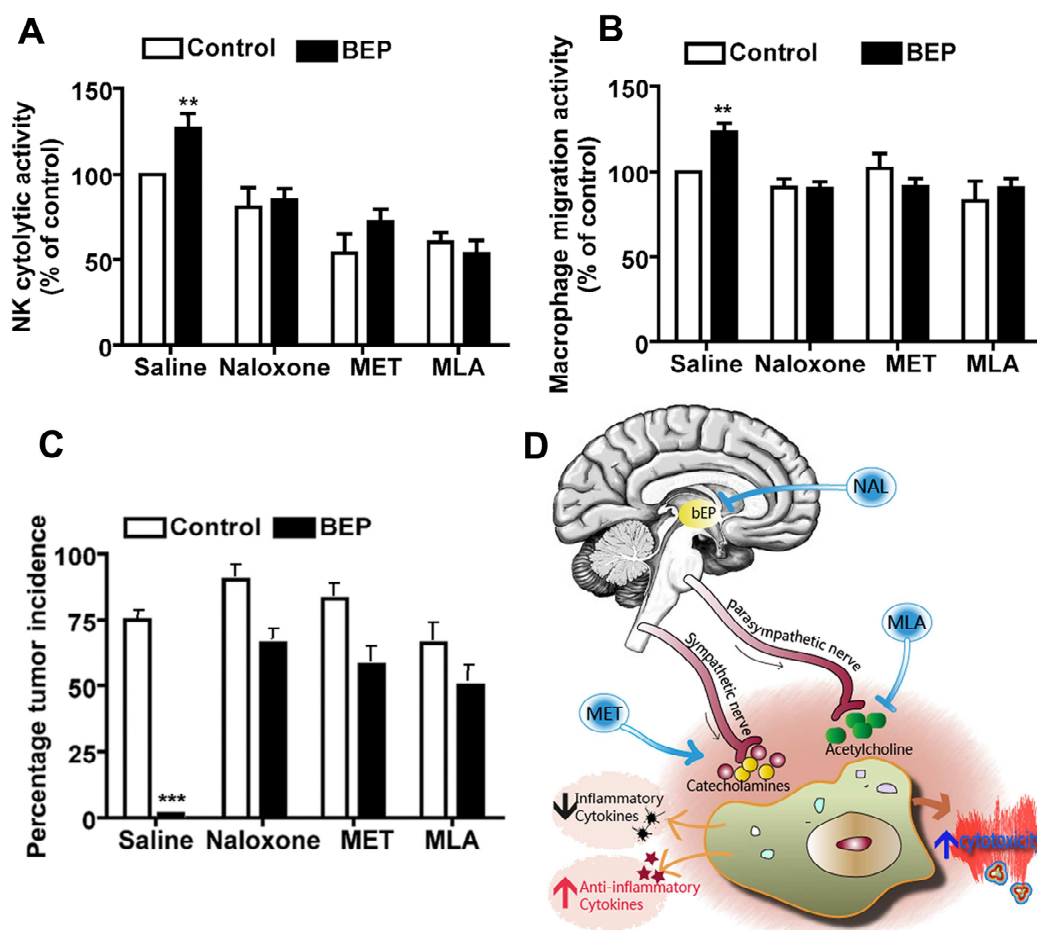
Figure 8: Autonomic mediation of the beneficial effect of β-EP transplants on immune activation and tumor cell metastasis in lung.

Animals were treated with the opiate blocker naloxone (NAL; 10 mg/kg body weight), β-receptors agonist metaproterenol (MET; 0.8 mg/kg body weight), nicotine acetylcholine receptor antagonist methyllycaconitine (MLA; 2.5 mg/kg body weight), or vehicle, were inoculated with MADB106 cells for determining immune and tumor clearance responses. **A**. PBMC-derived NK cells' cytolytic activity. **B**. PBMC-derived macrophage migration activity. **C**. Percentage of tumor incident as compared to control. $n = 12$ rats. [**], $P < 0.01$ vs. control of the similarly drug treated group. **D**. A hypothetical model showing how autonomic nervous system may mediate β-EP neuronal control of immune system.

3.4   Discussion

In situ, β-EP neurons originating from the arcuate nucleus terminate in the PVN and are known to regulate both stress axis and immune functions. The data presented here show that the

neural stem cell–derived β-EP neurons, when transplanted in the PVN, remained viable and increased endogenous opioid inhibitory tone to the HPA axis so that plasma corticosterone levels responded lower during stressful conditions like immune challenge. β-EP cell transplantation does not appear to affect general body growth or reproductive hormone profiles as the body weight and reproductive cyclicity of these rats were similar to those in controls. Within the context of immune-related function, *in situ* β-EP neurons in the hypothalamus are known to increase NK cell function via inhibition of sympathetic neurons to the spleen [113]; our present data are consistent with this (Fig. 8D). Furthermore, we observed for the first time that β-EP neurons were able to stimulate parasympathetic neurons to activate both NK cells and macrophages. Recently a role for parasympathetic neurons in immune activation has been revealed [323]. Studies have shown that decreased NK cells activity is associated with growth and progression of variety of cancers in animals [326-327] and humans [328-330], because NK-cells appear to represent a first line of defense against the metastatic spread of tumor cells [326]. In breast cancer patients, low NK cell activity seems to be heavily related with larger tumor growth [329] and also a predictive parameter of advanced disease (stages II, III, and IV) than in women with limited disease (stage I) [330]. At present, modulation of immune function especially enhancing NK cell activities, appears to be the most promising and new approach to cancer treatment. In this study, we showed here that supplementation of β-EP neurons, through transplants, prevents mammary tumor growth, progression, and metastasis. Importantly, when the β-EP transplants were given at the early stage of tumor development, many tumors were destroyed possibly due to increased innate immune activity and the surviving tumors lost their ability to progress to high-grade cancer due to β-EP cells' suppressive effects on inflammation-induced EMT regulators.

It is well known that inflammatory tumor microenvironment propel the migration and invasion of tumor cells through induction of EMT [309]. Hence, regulating inflammation and EMT may be a

potential novel approach to reverse the progression of tumor. Another remarkable effect of the β-EP transplantation was that it promoted the activation of the innate immune activity following tumor cell invasion to such an extent that tumor cell migration to another site was completely halted. The NK cells and macrophages are critical components of the innate immune system and play a vital role in host defense against tumor cells [331]. Hence, the increased level of innate immunity may have caused unfavorable conditions for cancer cell survival. In the β-EP cell–treated animals the lower inflammatory milieu that was achieved by the higher level of anti-inflammatory cytokines and the lower level of inflammatory cytokines may have also been involved in inhibiting cancer growth and transformation. Several studies have addressed the involvement and roles of the inflammatory chemokines and cytokines in mammary malignance [308-309]. Additionally, pharmacological modification of autonomic function significantly blocked the innate immune response and enhances the tumor cell metastasis and thus provides a plausible molecular mechanism for the protective role of β-EP neurons against the cancer progression and metastatic diffusion of mammary tumor cells. Our study not only identified the importance of stress maintenance in regulating immune function in cancer patients but also provided support for a potential therapeutic use of β-EP cell therapy for controlling mammary cancer and possibly other cancers.

CHAPTER 3

4.   **Chapter 3: in situ differentiation of β-EP via nanosphere-delivered dbcAMP**

increases β-EP production, alters stress response, and suppresses mammary cancer growth

4.1   Introduction

Body and mind interact extensively with each other. It has been found that stress is correlated with cancer progression, and measures that aimed at reducing stress were shown to be effective in inhibiting cancer growth [332]. β-endorphin (β-EP), an endogenous opioid polypeptide produced by neurons and immune cells, is known to have the ability to inhibit stress hormone production, produce analgesia and a feeling of well-being [18-19]. During stress, secretion of CRH and catecholamines stimulates secretion of hypothalamic β-endorphin (β-EP) and other POMC-derived peptides, which in turn inhibit the activity of the stress system [225]. Previous studies showed that β-EP in the hypothalamus enhances immune function and prevent cancer growth by stimulating NK cell cytolytic activity. The regulation of NK cell function is through acting directly on the spleen and by altering the neuroendocrine-immune system function [147, 333]. Previous work of our lab showed administration of β-EP peptide in the PVN increases NK cell function [223]. Perfusion of β-EP or δ-opioid receptor-specific agonists has been shown to stimulate NK cell cytolytic activity and lymphocyte proliferation [250-251], and these effects are blocked by intracranial administration of δ-specific antagonist [146]. NK cells are important mediators of cancer immune-surveillance [334]. This raises the question of whether increased β-EP production in the hypothalamus will increase the body's ability to control cancer growth.

Our lab has previously described a method of culturing β-EP neurons by *in vitro* differentiation of hypothalamic neuronal stem cells (NSCs) using an analog of cyclic adenosine monophosphate (cAMP), dbcAMP, and PACAP (pituitary adenylate cyclase-activating peptide) as inductive signals [221]. NSCs are multipotent cells that have the property of self renewal. Since neuronal

differentiation from NSC persists in the adult, and most of the neurons in the hypothalamus are derived from the proliferative neuroepithelium of the third ventricle [293], it is possible to induce NSC to differentiate into β-EP neurons *in vivo* by administering cAMP-activating agents into the third ventricle. To immobilize and ensure a slow release of cAMP and PACAP molecules, we used nanospheres as a carrier. Nanospheres are commercially made spherical particles, typically 13-20 nanometers (nm) in diameter. Each nanosphere can be functionalized with a defined number of certain molecules. As these nanospheres are injected into the third ventricle of the brain, they will stay in the site where they are injected, and slowly release the cAMP-activating agents they carry to the surrounding epithelium, therefore possibly stimulating the differentiation of β-EP neurons. Therefore we tested if cAMP-delivering nanospheres, after injection into the third ventricle, could induce the differentiation of neural stem cells into β-EP neurons, inhibit the activity of the stress axis, increase innate immune function, and inhibit cancer development.

We also tested this cAMP-delivering system in a fetal alcohol exposed rat model that is known to suffer from decreased β-EP production [20]. Lower numbers of β-EP neurons or peptide production have been also found in brains of patients with schizophrenia, depression, and obese patients [20-22], and these pathological conditions were correlated with higher incidences of cancers and infections [23-25]. Therefore, we tested the hypothesis that by injecting cAMP-delivering nanospheres, we could reverse the adverse effect of fetal alcohol exposure on stress level, immune function and cancer development.

3.2   Materials and Methods

Animals

Adult Sprague-Dawley and Fischer 344 rats were purchased from Charles River Laboratories (Wilmington, MA) and maintained in a controlled environment with a 12-hour light/dark cycle at the Bartlett Hall Animal Research Facility of our institute. All the animals were housed

individually, and allowed free access to regular rat chow, water, and maintained their normal physical activities throughout the study. Animal surgery and care was done in accordance with institutional guidelines and complied with NIH policy.

cAMP release test

Plain nanospheres and nanospheres that deliver 70 nmol and 350 nmol cAMP in 10 μL were purchased from Corpuscular Inc. (Cold Spring, NY). To test the efficacy of nanospheres in releasing cAMP, we put 10 μL nanospheres into 1 mL of artificial cerebrospinal fluid, and incubated at 37 °C. After 6 hours, nanospheres were spun down by centrifuging at 10000 g for 1 min, and supernatant were collected for analysis of cAMP concentration. Nanospheres were re-suspended in another 1 mL of ACSF, and incubated for 18 hours. Similarly, supernatants were removed at 24 hours, 48 hours and 72 hours and analyzed for cAMP concentration using ELISA kit (Enzo, Farmingdale, NY).

Nanosphere injection to animals

To determine the effect of cAMP-delivering nanosphere on hormone levels in rats, we used 18 adult male Sprague Dawley rats. Animals were anesthetized with sodium pentobarbital (50 mg/kg body weight, Butler Schein, Columbus, OH) and injected with 10 μL plain nanospheres (control) or nanospheres that deliver 70 nmol cAMP or 350 nmol cAMP in the third ventricle using a stereotactic instrument. To be specific, 10 μL of nanospheres were injected into the brain with the coordination of 2.0 mm behind the bregma, midline, and 8.0 mm below the cortex using a 10 μL Hamilton syringe. Each injection was over 5-min duration. After the injection, the cannula was left in place for 3 min to prevent nanospheres from backflow during the removal of the cannula. The cannula was then slowly removed over a 3-min period. The skin was closed with wound clips. Animals then received i.p. injection of bromodeoxyuridine (BrdU, 50 mg/kg body weight, Sigma) every day for 7 days. BrdU is an analog of Thymidine, and can be incorporated into dividing cells

and detected by BrdU antibody. 1 month after nanosphere injection, animals were sacrificed, and brains were collected for identification of β-EP neurons. Another 24 male Sprague Dawley rats were used for examining the effect of nanospheres during 2 weeks to 2 months after injection. Animals were injected with nanospheres that deliver 70 nmol cAMP in 10 μL 2 months, 1 month and 2 weeks before the day of sacrifice. Control animals were kept intact until sacrifice. Hypothalamic tissues, pituitary and blood plasma were collected for detection of hormones.

Tumor induction and characterization

To determine the effect of cAMP-delivering nanosphere injection on mammary tumor growth and progression, 50-day old ovary intact virgin Sprague-Dawley rats were injected intraperitoneally (i.p.) with a dose of MNU (50mg/kg body weight, Sigma). Six weeks after the MNU injection, animals were anesthetized with sodium pentobarbital (50 mg/kg body weight, i.p.; Butler Schein, Columbus, OH) and injected with plain nanospheres (control) or 70 nmol cAMP-delivering nanospheres in the third ventricle using stereotactic instrument as previously described. No tumors were detected at this time. Beginning 1 week after surgery, when the animals were recovered from the brain surgery, animals were weighed and palpated every week to check for tumor growth. Tumor length and width were measured with a caliper, and tumor volumes were calculated as ½ x (length x width)$^{3/2}$. Sixteen weeks after the MNU injection, animals were sacrificed, tumors were collected, and slices of tumors were immersed in formalin and processed for histology staining. Fixed tissue was dehydrated, cleared, and paraffin infiltrated overnight using a tissue processor. The tissues were paraffin embedded, sectioned into 5 μm thick slices and placed on slides. One slide from each tissue was stained with hematoxylin and eosin (H&E) to evaluate tissue histology and tumor pathology.

Metastasis study

We used the MADB106 cells, a Fischer 344 rat mammary adenocarcinoma cell line maintained in RPMI medium containing 10% fetal bovine serum, and were harvested by treating with trypsin+EDTA (Sigma, St. Louis, MO). Female Fischer-344 rats were injected with control or cAMP-delivering nanospheres at 60 days of age. After one month, these nanosphere-injected rats were inoculated with MADB106 tumors cells (100,000 cells/0.2 mL/rat) into the jugular vein under anesthesia of isofluorane. Wounds were closed with a surgical clip. Rats were sacrificed at 6 weeks after tumor inoculation, and the whole body of each animal was inspected for the presence of visible tumors. Tumors were only located in the lungs in most animals, and at the inoculation site in a few animals. Lungs were collected, fixed in formalin, embedded with paraffin, and sectioned for H&E staining. Stained slides were examined under the microscope for the verification of the presence of tumor.

Immune reaction after tumor cell inoculation

We checked immune reaction to tumor cell inoculation in animals after they received injection of control or cAMP-delivering nanospheres for 4 weeks. Animals were anesthetized with isofluorane and inoculated with MADB106 cells (100,000/0.2 mL/rat) into the jugular vein. After 24 hours, animals were sacrificed, and blood and spleens were collected. Peripheral blood mononuclear cells (PBMCs) were collected from the blood, and splenocytes were collected from the spleens. PBMCs and splenocytes were used for NK cell cytolytic assay and macrophage migration assay (Cell BioLab, San Diego, CA), as well as stained by FITC-conjugated antibodies for CD161a and CD11b/c for flow cytometry.

Plasma analysis of hormones

Plasma was analyzed for corticosterone levels by a competitive ELISA (ImmunoDiagnostic Systems) according to manufacturer's direction. All samples were run on one 96-well plate.

Immunohistochemistry

Six brains from each treatment groups were cryosectioned at 20 μm in thickness, and sections were placed on a glass slide (Superfrost plus, VWR, Bridgeport, NJ) and were kept frozen at -80°C until staining. Brain sections were collected from plate 18 to plate 23 of the stereotaxic atlas (Paxinos and Watson, 1989) to cover the whole paraventricular nucleus and arcuate area of the hypothalamus. Every fifth section was used for staining each peptide. Brain slides were fixed with 4% paraformaldehyde for 20 min, and then washed with PBS twice. They were then fixed with methanol for 10 min and washed again with PBS. The brain sections were then blocked with 2.5% horse serum for 1 hour and incubated with mouse anti TH (1:500) antibody for 2 hours or rabbit anti CRH antibody (1:2000) overnight. For double staining, sections were incubated with rabbit anti β-EP (1:2000, invitrogen) antibody and mouse anti BrdU antibody (1:2000) for overnight. The sections were then washed twice and incubated with Alexa anti-mouse IgG (1:500) and FITC anti-rabbit IgG (1:500) for 45 min. Stained slides were mounted with DAPI and pictures were taken by confocal microscope with 20x objective lens (Nikon EZ-C1 3.60 build 770, Gold version). Cell numbers on each section were manually counted and the sum of total cell number from all sections of one brain was calculated.

Measurement of cytotoxicity

The cytotoxicity of PBMC and splenocytes at each time point was determined by calcein AM assays using YAC-1 murine lymphoma cells (ATCC, Manassas, VA) as target cells. YAC-1 cells were grown and maintained in RPMI 1640 medium containing 1% antibiotics (Sigma-Aldrich) and 10% FBS (Sigma-Aldrich). YAC-1 cells were washed and incubated with 5 mM calcein AM (Sigma-Aldrich) in serum-free RPMI 1640 medium for 40 min at 37 °C. Labeled YAC-1 cells were washed and plated into U-bottom 96 well plates (BD Falcon, Franklin Lakes, NJ) at 5×10^4 cells per well. PBMC or splenocytes incubated with IL-2 (100 ng/mL, R&D Systems, Las Vegas, NV) for 12 h at 37 °C were then added at various E:T ratios in triplicate. YAC-1 cells in RPMI alone were used to determine spontaneous calcein release, whereas maximal release was achieved

by lysing target cells with 0.1% Triton X-100. Cells were incubated at 37 °C for 4 hours, and fluorescence in the supernatant was measured using a fluorescence plate reader (Tecan). The percent cytotoxicity for each sample was calculated as follows: % cytotoxicity= [(experimental well – spontaneous well)/(max lysed well – spontaneous well) x 100. The percentage at each E:T ratio were converted to Lytic Units per 10^7 effector cells and based on 20% specific cell lysis [335].

Statistics

Differences in average tumor incidence, tumor volume and tumor number were assessed using two-way ANOVA followed by Bonferroni post-test. β-EP cell number, β-EP + BrdU doubled stained cell number, and β-EP and stress hormone levels were analyzed using one-way ANOVA. CRH and TH neuronal numbers, NK cell and macrophage activities and population were analyzed using t-test. To evaluate tumor type and lung tumor incidence, Chi-square tests were performed. A $p<0.05$ was considered significant.

3.3 Results

Nanosphere-delivered cAMP increased β-EP level in the hypothalamus, and decreased levels of stress hormones

We have previously shown that cAMP induces differentiation of hypothalamic neuronal stem cells *in vitro*. It is known that neuronal stem cells are located in the epithelium of ventricles in adult brain. Therefore we tested the possibility of inducing differentiation of those stem cells *in vivo* by injecting cAMP analog to the 3rd ventricle. To effectively deliver cAMP into the third ventricle of the brain, we took advantage of a novel carrier system, in which the cAMP molecules are attached to nanospheres and get slowly released locally. An *in vitro* condition that mimicked the *in vivo* environment was used to test the ability of cAMP-carrying nanospheres in releasing cAMP in the 3rd ventricle. Three types of nanospheres were used: 1) control nanosphere with

nothing attached (Control); 2) nanosphere that has 70 nmol of dbcAMP attached covalently in a volume of 10 μL (70 nmol cAMP); and 3) nanophere that has 350 nmol of dbcAMP attached covalently in a volume of 10 μL (350 nmol cAMP). When incubated in ACSF at 37 °C, dbcAMP-delivering nanospheres released a surge of cAMP within the first 6 hours. Afterwards, the release of cAMP decreased to about 2 nmol/24h in the case of low-dose nanosphere, and about 10 nmol/24h in the case of high-dose nanosphere (Fig. 9A).

In order to test the efficacy of nanosphere-delivered cAMP in inducing the *de novo* production of β-EP neurons *in vivo*, we implanted nanospheres, and then injected the rats with BrdU for 7 days. BrdU was incorporated into the dividing cells, enabling us to detect the cells that were newly produced after nanosphere injection. Double staining with β-EP and BrdU antibodies identified not only that there was an increase of β-EP neuronal cell numbers after injection of cAMP-delivering nanospheres (Fig. 9B, E), but that a portion of the increased β-EP neurons are newly arose from divided cells within 7 days after nanosphere injection (Fig. 9C, E). An assay that detects β-EP peptides in the hypothalamic tissue also showed an increase of β-EP content in cAMP injected rats (Fig. 9D). Since a higher dose of cAMP on the nanosphere didn't show a significantly higher effect on the increase of β-EP production compared to the lower dose of cAMP, 70 nmol dose of cAMP appears to produce the maximal effect.

As an endogenous opioid peptide, β-EP is known to reduce stress. To examine the effect of cAMP-delivering nanospheres in regulating stress hormones in the hypothalamus, we stained brain slices with CRH. We also stained brain slices with TH (tyrosine hydroxylase) antibodies as a control, and counted the total number of immunostaining positive cells in the whole hypothalamus. Our results showed that the number of CRH positive cells in the PVN area was decreased in cAMP nanosphere injected rats (Fig. 9F, G), indicating a suppressed HPA axis. However, we didn't see a significant change in the number of neurons stained positive for TH in the arcuate neuclus (Fig. 9H, I).
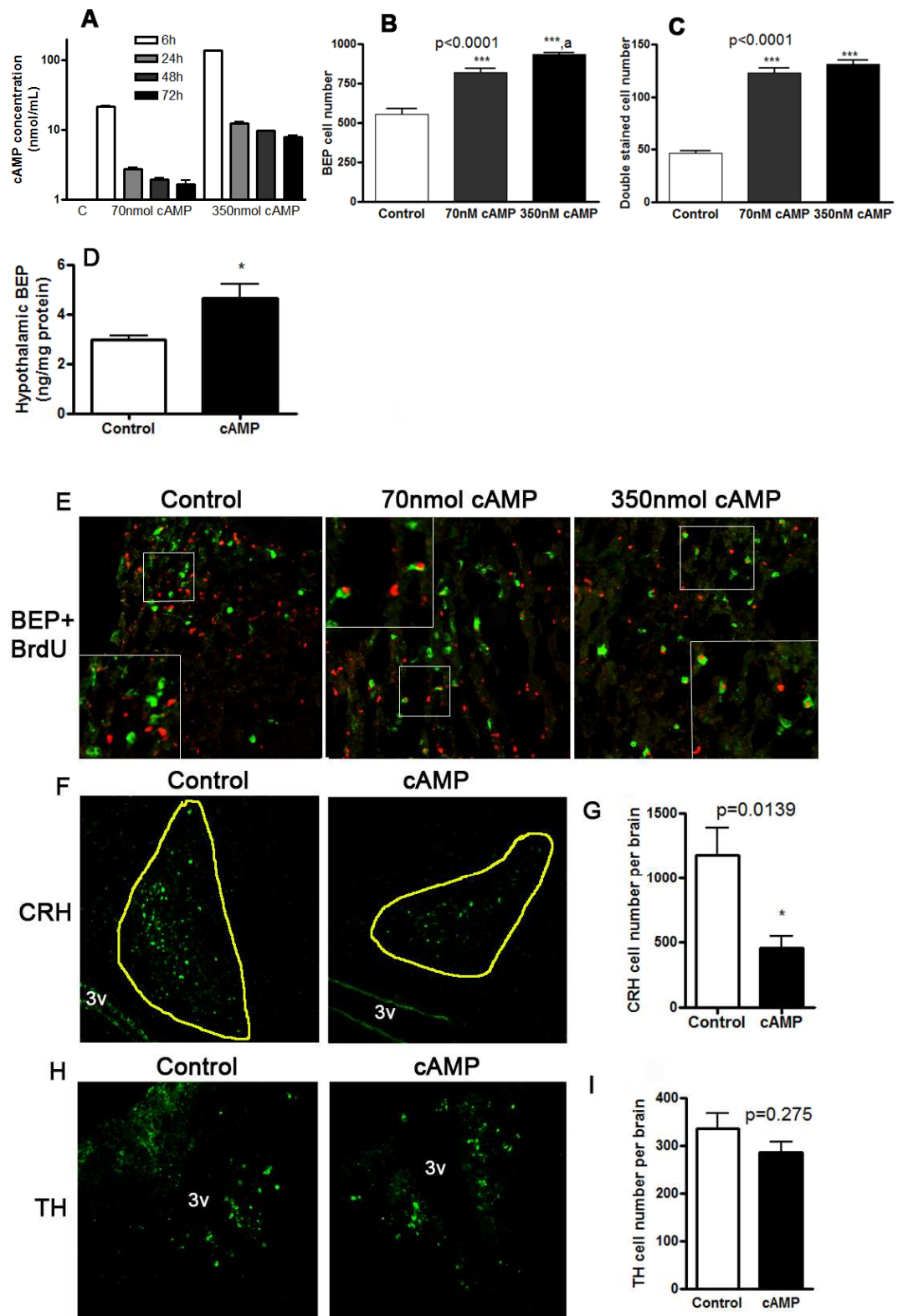
Figure 9: Nanosphere-delivered dbcAMP injection increased differentiation of NSCs into β-EP neurons in the hypothalamus.

Animals were injected with control or dbcAMP-delivering nanospheres into the third ventricle, and were sacrificed after 1 month to check for neuronal numbers in the brain by histo-immuno-staining, or for peptide concentration by ELISA and protein assay. A. Amount of dbcAMP released by 10 μL nanospheres at different time points. B. Total numbers of β-EP immune-staining positive neurons in the arcuate nucleus of nanosphere-injected brains. C. Numbers of β-EP +BrdU double stained neurons in the arcuate nucleus of nanosphere-injected brains. D. β-EP peptide levels in the hypothalamus. E. Representative fluorescent microscope photograph of β-EP +BrdU double stained neurons (Green: β-EP, Red: BrdU). F. Representative photograph of the CRH staining. G. Total count of CRH positive neurons in the PVN. H. Representative photograph of the TH staining. I. Total count of TH positive neurons in the arcuate nucleus. B and C were analyzed using one-way ANOVA, and D, G and I were analyzed using t-test. *: $p<0.05$ comparing to control group. ***: $p<0.001$ comparing to control. a: $p<0.05$ comparing to 70 nmol cAMP group.

Previous experiments were conducted at 1 month after nanosphere injection. Our next questions are how soon the injection of cAMP-delivering nanosphere can be effective, and how long will the effect last. Rats were injected with nanospheres, and sacrificed after 2 weeks, 1 month and 2 months. Measurement of hormone levels using ELISA and POMC mRNA levels using real-time PCR showed that injection of cAMP-delivering nanospheres into the third ventricle significantly increased both β-EP peptide and content of POMC mRNA in the hypothalamus to the same extent from 2 weeks to 2 months after nanosphere injection (Fig. 10A, B). Hypothalamic CRH (Fig. 10C), pituitary ACTH (Fig. 10D), and plasma corticosterone contents (Fig. 10E) were all decreased from 2 weeks to 2 months after nanosphere injection, possibly due to the inhibitory effect of β-EP on the stress axis. Besides the HPA axis, activity of the SNS was also suppressed by injection of cAMP-delivering nanospheres, as shown by decreased plasma levels of epinephrine (Fig. 14H) and norepinephrine (Fig. 14I)
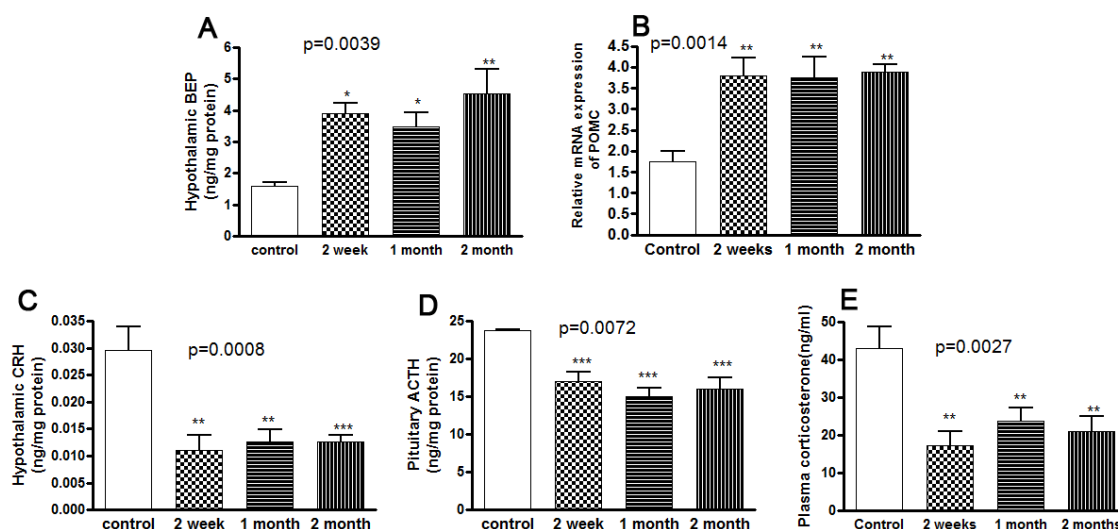
Figure 10: Time course of the effect of nanosphere-delivered dbcAMP on neuronal peptides and stress hormones.

Animals were injected with nanospheres and sacrificed after 2 weeks, 1 month and 2 months. A. β-EP peptide level in the hypothalamus. B. POMC mRNA level in the hypothalamus. C. CRH peptide level in the hypothalamus. D. ACTH concentration in the pituitary. E. Plasma concentration of corticosterone. Data were analyzed using one-way ANOVA. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ all compared to control group.

Injection of cAMP-delivering nanosphere inhibited carcinogen-induced mammary cancer and

mammary cancer cell metastasis

Many animal experiments and clinical observation supports the concept that chronic stress increases cancer incidence, malignancy and growth rate, while managing of stress promotes immune function that fights against cancer. Since injection of cAMP-delivering nanospheres into the 3rd ventricle was shown to suppress the levels of stress hormones, we tested the effectiveness of injection of these nanospheres on MNU induced mammary cancer in rats. Rats were injected with nanospheres 4 weeks after MNU injection. We found that injection of cAMP-delivering nanospheres significantly reduced tumor incidence (Fig. 11B), tumor number (Fig. 11C) and tumor volume (Fig. 11D) in MNU-induced mammary cancer. Tumors from the study were classified by histopathological analysis (Fig. 11E), which revealed that control animals had more

adenocarcinoma, while cAMP-delivering nanosphere injected animals had mostly benign adenoma.

To determine the effect of cAMP-delivering nanospheres on metastasis of mammary cancer, we used MADB106 mammary cancer cell line, which was syngeneic to Fischer rats and widely used in lung metastasis studies. Four weeks after inoculation of MADB106 cells through jugular vein, 70% to 80% of inoculated control-nanosphere-injected rats showed visible tumor granules in the lungs (Fig. 11F, H). On the contrary, only 20% of inoculated cAMP-delivering-nanosphere-injected rats had foci of tumor in the lung. Furthermore, this beneficial effect of cAMP-delivering nanospheres on preventing tumor metastasis was reversed by injection of Naloxone, a competitive antagonist of μ-opioid receptor. This observation indicated that the beneficial effect of nanospheres was acting through opioid receptors, which are the primary binding sites of β-EP peptide. Tumor growth in the lung was confirmed by histological examination of formalin-fixed lung tissue. Plasma samples drawn from these rats at the time of tumor inoculation were analyzed for concentration of corticosterone as an indicator of stress level. It is shown that cAMP-delivering nanospheres decreased the corticosterone level, while corticosterone levels were increased to normal in Naloxone-treated animals (Fig. 11G).
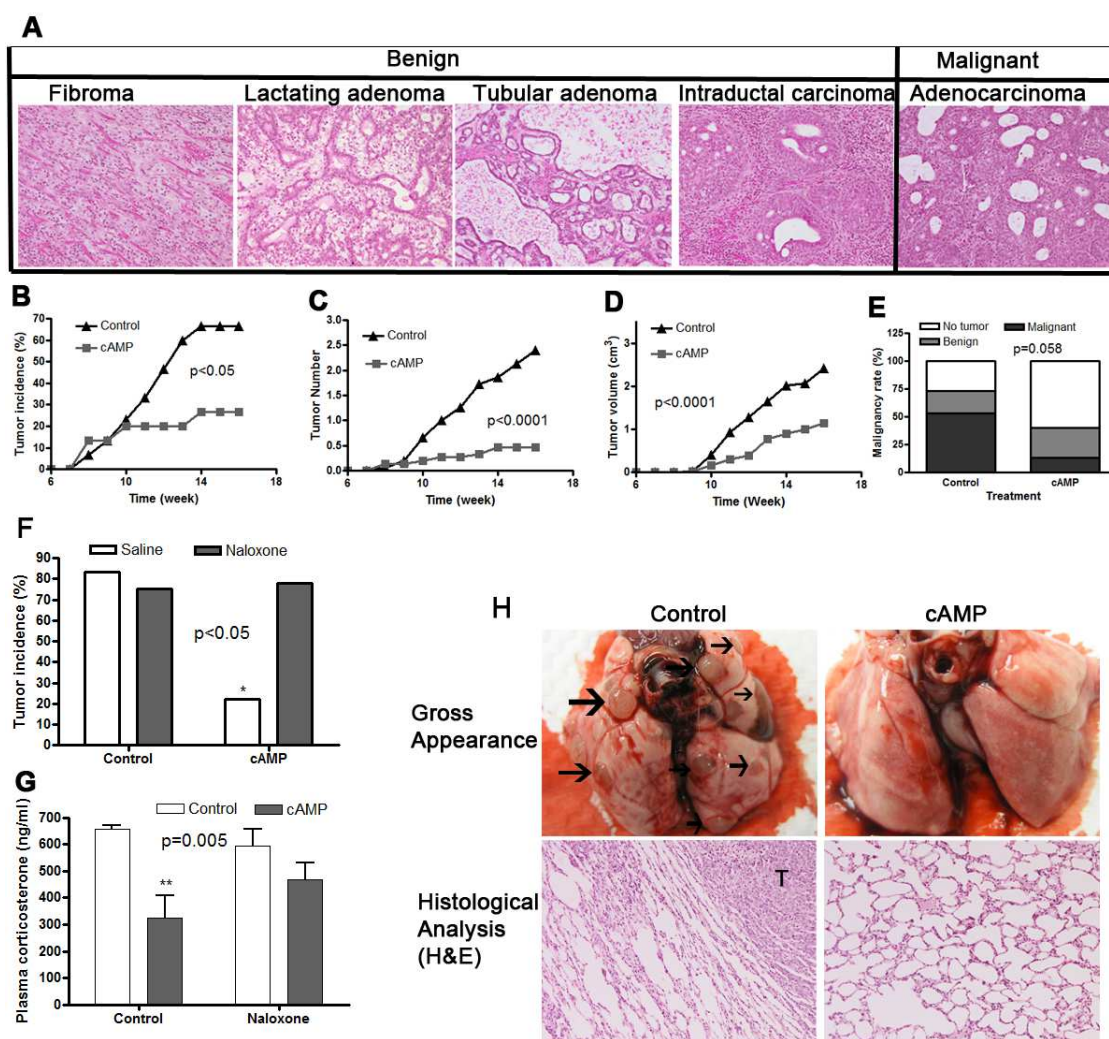
Figure 11: Effect of dbcAMP-delivering nanosphere injection on carcinogen-induced mammary cancer and mammary cancer cell metastasis.

For carcinogen-induced mammary cancer, animals were injected with MNU at 49 days old. After 4 weeks, they were injected with control or dbcAMP-delivering nanospheres. Animals were palpated every week until 16 weeks after MNU injection. A. Representative light microscope H&E staining photographs of different histological tumor types developed in our model. B. Percentage of rats with tumor in each group (tumor incidence). C. Average tumor number in each group (total tumor number in the group divided by number of animals in the group). D. Average tumor volume in each group (total tumor volume divided by number of animals in the group). E. Tumor malignancy rate as determined by histological evaluation (percentage of each type of tumors developed in each treatment group). For cancer cell metastasis study, animals were injected with control or dbcAMP-delivering nanospheres. After recovery these animals were treated with saline or naloxone, and at the same time inoculated with 100,000/0.2ml/rat MADB106 cells into the jugular vein. These animals were sacrificed after 6 weeks to check for tumor growth. F. Percentage of animals in each group that developed tumor in the lung. G. Plasma corticosterone concentration at the time of tumor inoculation. H. Representative photographs of a lung with tumor in control group, and

normal a healthy lung in the group injected with dbcAMP-delivering nanospheres. B~D were analyzed with two-way ANOVA, E and F were analyzed with Chi-square test, and G was analyzed with one-way ANOVA. *: p<0.05, **: p<0.01.

Nanosphere-delivered dbcAMP increased innate immune functions.

Since nanosphere-delivered dbcAMP increased β-EP peptide production in the hypothalamus, and since β-EP in the hypothalamus was shown to increase innate immune function [113, 146, 250], which is critical for inhibition of cancer development, we hypothesize that the inhibition of mammary cancer growth, and the elimination of metastatic tumor cells in the dbcAMP-nanosphere injected rats may have been caused by increased innate immune function. We checked the immune cell activities 24 hours after inoculation of MADB106 mammary cancer cells, and found that dbcAMP-nanosphere injected animals had higher macrophage migration activity cytolytic activity in both PBMC and splenocytes (Fig. 12A). dbcAMP-nanosphere injected animals also had increased NK cell and macrophage population in both PBMC and splenocytes (Fig. 12B, C). Additionally, examination of spleen cytokines in another mammary cancer experiment showed that dbcAMP nanosphere injected animals had increased IFN-γ (Fig. 12D) and granzyme B (Fig. 12E), indicating an increased function of NK cells.

Together, these data suggest that injection of cAMP-delivering nanospheres into the 3rd ventricle significantly inhibited metastasis of mammary cancer cells into the lung, and this effect was possibly mediated through increase of β-EP, decrease of stress, and increased innate immune functions in the animals.
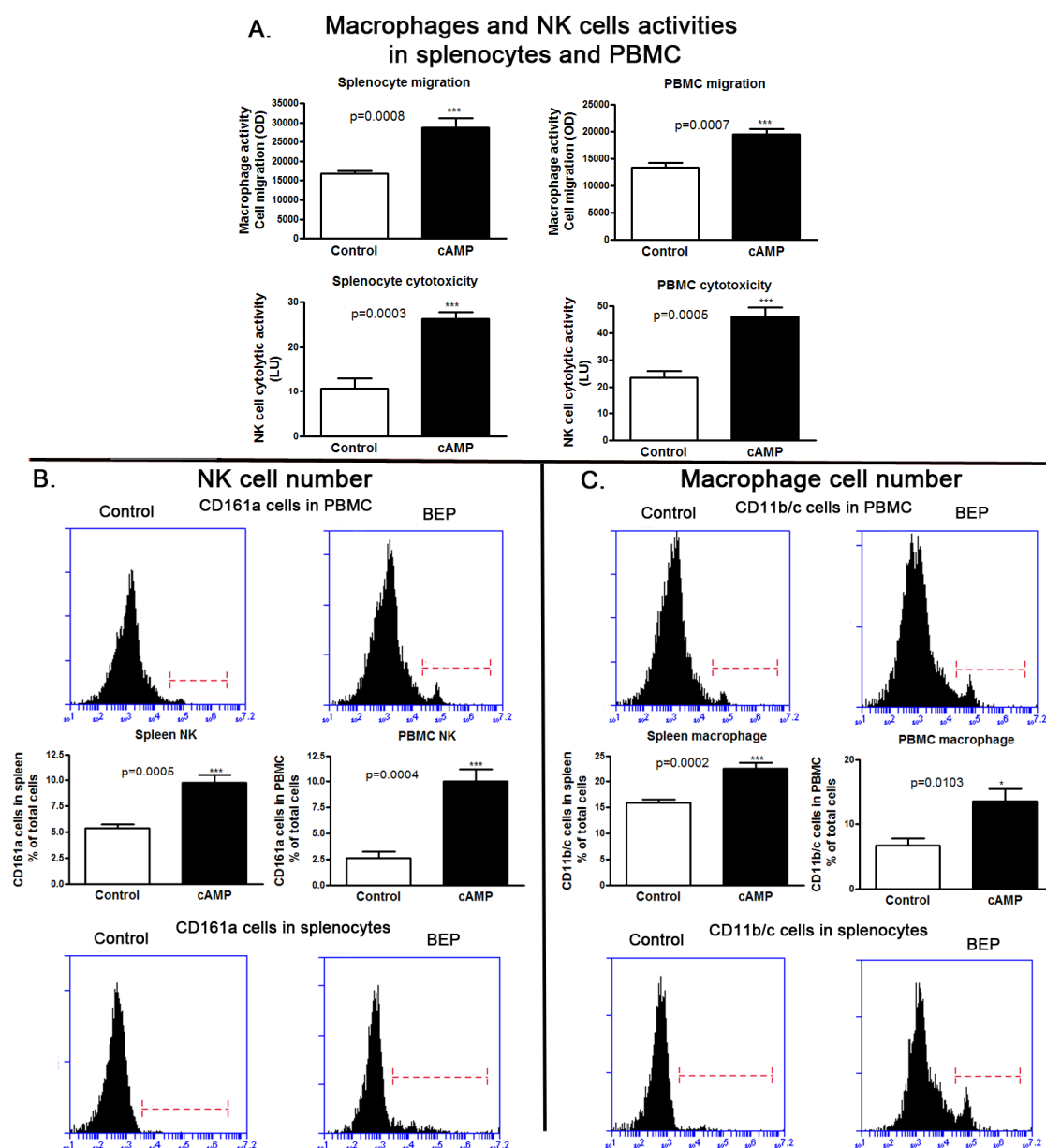
Figure 12: Evaluation of effects of nanosphere-delivered dbcAMP on NK cell and macrophage activities.

Animals were injected with control or dbcAMP-delivering nanospheres. After recovery from the brain surgery, they were inoculated with MADB106 cells into the jugular vein, and sacrificed after 24 hours. PBMC from the blood and splenocytes from the spleen were collected in order to test NK cell activity and macrophage activity. A. Macrophage migration activity and NK cell cytolytic activity in PBMC or splenocytes 24 hours after tumor cell inoculation. B. NK cell (as indicated by CD161a staining) population in PBMC and splenocytes. C. Macrphage (as indicated by CD11b/c population in PBMC and splenocytes. Data were analyzed using t-test.*: p<0.05, ***: p<0.001.

3.4 Discussion

β-EP is a key regulator of the stress response of the body. It inhibits both the HPA axis and sympathetic nervous system through negative feedback [223, 225]. The present study demonstrates that cAMP delivered by nanospheres is able to inhibit mammary cancer growth in both normal animals and fetal alcohol exposed animals, possibly by increasing the β-EP neuropeptide production in the hypothalamus and increasing innate immune function of the body. The increased immune function may be due to the decreased activity of the stress axis, which may be inhibited by the increased β-EP production.

Our lab has previously reported that β-EP neurons can be differentiated from hypothalamic NSCs *in vitro* by cAMP stimulating reagents. After transplantation of these neurons into the PVN in the hypothalamus, these β-EP neurons can be integrated into this site, and produce β-EP peptide hormone. Rats with these neuronal transplants have reduced susceptibility to carcinogen-induced mammary cancer and prostate cancer, and are resistant to lung metastasis of mammary cancer cells [221, 336]. These effects are possibly mediated through an increased NK cell activity, reducing the body's inflammatory milieu, decreased sympathetic nervous system activity, and increased activity of parasympathetic nervous system. Our lab has also shown that *in vitro*-produced β-EP neuronal transplants can eliminate NK cell functional deficiency and recover the decrease of IFN-γ in fetal alcohol exposed rats [113]. Instead of directly transplanting neurons into the PVN, here we induced the differentiation of hypothalamic NSCs to β-EP neurons *in vivo* using the same drug dbcAMP slowly released in the 3rd ventricle. This method increased the β-EP production in the hypothalamus and also decreased the mammary cancer incidence in treated animals, which is consistent with our previous findings with the β-EP neuronal transplantation model. This new method using nanosphere-delivered dbcAMP may be beneficial comparing to the previous neuronal transplant model in that the differentiated neurons are generated from the animal itself, and will not be rejected by the immune system.

The hypothalamus is an important portion of the brain in that it links the nervous system with the endocrine system, acts as a key regulator of the endocrine system, and regulates the autonomic nervous system. Naturally, β-EP neuronal cell bodies are localized in the arcuate nuclei of the hypothalamus, including many areas of the hypothalamus and limbic system [221], and its terminals are distributed throughout the central nervous system. β-EP neurons innervate CRH neurons in the PVN and inhibit CRH release [222]. In our study, the newly differentiated β-EP neurons were observed in the arcuate nucleus in the hypothalamus. Together with the increased number of β-EP neurons in the hypothalamus, we also observed a decrease of CRH production in the PVN. Therefore it is plausible that these β-EP neurons formed connections with other parts of the central nervous system, as the normal developing neurons would do, and controlled the activity of CRH neurons, SNS and PSNS in the PVN.

We have previously shown that dbcAMP acts as a neurotropic factor for immature β-EP neurons [42]. Also dbcAMP has been found to be effective in enhancing the differentiation of NSCs to neurons in culture and transplantation, and could be used in axonal regeneration in traumatic spinal cord injury [288, 337]. The hypothalamus consists of several groups of hormone-secreting neurons that are critical for various neuroendocrine functions [338]. Most of the neurons in the hypothalamus are derived from the proliferative neuroepithelium of the third ventricle [293]. Zahir et al. screened several factors for promoting neurogenesis from adult rat subventricular zone-derived NSCs and reported that dbcAMP enhanced neuronal differentiation after one week of exposure in culture [291]. This is thought to be primarily mediated through the PKA pathway [282], which causes the up-regulation of cAMP-responsive element binding protein (CREB), which is an important transcription factor in regulating neuronal activity [339]. In our previous study we treated hypothalamic NSCs with dbcAMP, and the majority of differentiated neurons showed β-EP positive immune-staining. In this study, after cAMP-delivering nanosphere injection, we observed the increase of β-EP positive neurons. We also checked the number of

other neurons that are localized in the acuate nucleus, such as TH neurons, and didn't find significant change in neuronal number. This result is in accordance with our previous observation, and indicates that dbcAMP treatment seems to induce the differentiation of hypothalamic NSCs specifically into β-EP neurons.

MADB106 mammary adenocarcinoma cell line is sensitive to NK cell cytotoxicity and therefore widely used in lung metastasis study as an indicator of innate immune function. MADB106 tumor cells metastasize only to the lungs [340]. Dr. Ben-Eliyahu's lab has previously shown that lung tumor retention of these MADB106 cells is increased by environmental and psychological stress, through the elevation of catecholamines from the sympathetic nervous system [341]. In accordance with their findings, here we show that injection of the cAMP-delivering nanospheres significantly reduced the level of epinephrine and norepinerphrine, increased NK cytotoxicity, and reduced the incidence of MADB106 cell colonization in the lung. This result indicates that the mechanism of dbcAMP treatment may be mediated through sympathetic nervous system and innate immune activity.

In conclusion, we have developed a novel method to induce the differentiation of NSCs to a specific type of endocrine neurons *in vivo*. These endocrine neurons are able to inhibit mammary cancer development in rats, possibly by inhibiting stress hormone production and by enhancing innate immune activity. Our method may be promising in developing therapy for treating mammary cancer and possibly other types of cancers.

CHAPTER 4

5   Chapter 4: β-EP neuronal transplantation and in situ differentiation of β-EP via nanosphere-delivered dbcAMP alters immune function and inhibits mammary cancer progression in fetal alcohol exposed rats.

5.1   Introduction

Fetal alcohol exposure occurs to the fetus when pregnant women drink alcohol. It causes a series of abnormalities collectively known as FASDs [26]. The prevalence of FASD is estimated to be 1 in every 100 live births, and it is the leading known cause of mental retardation in the western world [27]. Patients with FASDs were found to have numerous behavior changes, such as depression, anxiety, hyperactivity, and inability to cope with stressful situations [6]. Fetal alcohol exposed children were shown to have higher level of cortisol in the blood [5], and the same effects were also observed in animal models of FASD [88-89], indicating defects in stress regulation caused by fetal alcohol imprinting. Fetal alcohol exposure also causes immune deficiencies in both human and lab animals [8, 115, 127]. Children with fetal alcohol exposure often show defects in host defense and higher incidence of infections, as well as lower immune cell count and reduced mitogen-stimulated proliferative responses of leukocytes [8]. Additionally, fetal alcohol exposed children were found to have increased incidence of different types of malignant neoplastic diseases, such as rhabdomyosacoma of urinary bladder and prostate [136], myeloid leukemia [11], and testicular cancer [12]. Fetal alcohol exposure was also shown to increase susceptibility to carcinogen-induced mammary cancer using rats as an animal model [9-10].

β-endorphin (β-EP) neurons in the hypothalamus play an important role in regulation of stress and immune functions. These neurons reside in the arcuate nucleus, and innervate the paraventricular nucleus (PVN) where they inhibit the activities of corticotrophin-releasing

hormone (CRH) neurons and sympathetic nervous system (SNS) [207]. Central administration of β-EP was shown to increase immune function, such as NK cytolytic activity and lymphocyte proliferative response to mitogens [146, 251]. It was found that fetal alcohol exposed rats have reduced β-EP production [20]. Lower numbers of β-EP neurons or peptide production have been also found in brains of patients with schizophrenia, depression, and obese patients [20-22], and these pathological conditions were correlated with higher incidences of cancers and infections [23-25]. These facts indicate that the defective immune function and increased incidence of cancer in fetal alcohol exposed patients may be related to the decreased β-EP neuronal numbers in the hypothalamus.

We have recently reported that by transplanting *in vitro* produced β-EP neurons into the PVN, we could prevent the growth of carcinogen-induced prostate cancer [221]. Using the same method, we also inhibited the hyper-active stress axis in fetal alcohol exposed animals, and increased their immune function [113]. We have also previously shown that transplantation of β-EP neurons suppressed mammary cancer growth and metastasis (Chapter 2). What is not known is whether this β-EP transplantation will help with the increased incidence of cancer in fetal alcohol exposed animals. Therefore here we further tested whether transplantation of β-EP neurons will help with the inhibition of mammary cancer development and metastasis in fetal alcohol exposed rats. We also tested the previously described dbcAMP-delivering system in a fetal alcohol exposed rat model for its efficacy in regulating stress, immune function and inhibition of mammary cancer.

5.2   Materials and Methods

Fetal alcohol exposure

Female Sprague Dawley rats were mated with males and their vaginal smears were checked every morning. The presence of sperm in the vaginal smearing indicated mating and that day was designated as gestational day 1 (GD1). One gestational days GD7-GD21, pregnant rats were fed

daily chow ad libitum (AD), a liquid diet (BioServ Inc., Frenchtown, NJ) containing alcohol (AF) or pair-fed with an isocaloric liquid control diet (PF, with the alcohol calories replaced by maltose-dextrin). The concentration of ethanol varied in the diet for the first 4 days from 1.7 to 5.0% v/v to habituate the animals with the alcohol diet. After this habituation period, animals were fed the liquid diet containing ethanol at a concentration of 6.7% v/v. At postnatal day 2 (PD2), AF and PF pups were cross-fostered by untreated lactation AD dams to prevent any compromised nurturing by the AF and PF moms. Litter size was reduced to 8 pups per dam. Pups were weaned on PD21, and housed by sex until the injection of MNU, when they were individually housed. MNU injection was the same as previously described. Rats were then palpated weekly from 5 weeks after MNU injection. These animals were used in both the β-EP transplantation study and the nanosphere study.

β-EP cells preparation and transplantation

Same as described in Chapter 2.

Mammary cancer treatment paradigm

In the β-EP study, rats that were newly found to have a tumor with a diameter larger than 0.5 cm were randomly transplanted with either cortical neurons as control or *in vitro* differentiated β-EP neurons (20,000/6 μL/per PVN) into both PVN. Animals continued to be palpated for 14 weeks, or until one dimension of the tumors reached 3 cm. In the nanosphere study, rats that were newly found to have a tumor with a diameter larger than 0.5 cm were injected with either plain nanospheres (control) or nanospheres containing 70 nmol dbcAMP (cAMP), and continued to be palpated until 10 weeks after the discovery of tumor or until tumor diameter reached 3 cm. Then the rats were sacrificed, and tumors were collected for histology diagnosis, spleens were collected for cytokine mRNA detection, and plasma samples were collected for measurement of stress

hormones. Tumor growth was measured using a caliper, and the volumes of tumors were calculated as ($\pi/2$ x Length x Width^2).

Lung metastasis study

Fetal alcohol exposed female rats were also transplanted with cortical neurons as control or *in vitro* differentiated β-EP neurons (20,000/6 μL/per PVN) into both PVN, and injected with 100,000 MADB106 mammary carcinoma cells suspended in 0.2 mL of RPMI medium into the jugular vein. At the time of tumor inoculation, 1 mL of vein blood was drawn into a 1 mL syringe containing 0.05 mL EDTA (100 mg/mL) for the test of plasma corticosterone level. These animals were sacrificed after 4 weeks. Tumor growth on the surface of the lungs was counted as an indicator of extent of metastasis.

Nanosphere injection to animals

Animals were anesthetized with sodium pentobarbital (50 mg/kg body weight, Butler Schein, Columbus, OH) and injected with 10 μL plain nanospheres (control) or nanospheres that deliver 70 nmol dbcAMP (cAMP) in the third ventricle using stereotactic instrument. To be specific, 10 μL of nanospheres were injected into the brain of animals (about 300 g body weight) with the coordination of 2.0 mm behind the bregma, midline, and 8.0 mm below the cortex using a 10 μL Hamilton syringe. Each injection was over 5-min duration. After the injection, the cannula was left in place for 3 min to prevent nanospheres from backflow during the removal of the cannula. The cannula was then slowly removed over a 3-min period. The skin was closed with wound clips.

RNA extraction and PCR

Total RNA was isolated from ~30 mg spleen tissue, using RNeasy Mini Kit (Qiagen). With the Superscript III First-Strand Synthesis SuperMix (Invitrogen) for RT-PCR, 100 ng total RNA was reverse transcribed and relative quantification of mRNA levels was performed by real-time RT-

PCR (SYBR Green; Applied Biosystems), using Applied Biosystems 7500 fast real-time PCR system. The following primer sequences were used. Perforin F: 5'-GCATCGGTGCCCAAGCCAGTC-3', R: 5'-GCCAGCGAGCCCCTGCTCA-3'; Granzyme B F: 5'- CGTGCATCAGAAGTGGTGTTG-3', R: 5'- GAGGCTGTTGTTACACATCCGG-3'; IFN-γ F: 5'- AGAGCCTCCTCTTGGATATCTGG-3', R: 5'- GCTTCCTTAGGCTAGATTCTGGTG-3'; TNF-α F: 5'-CCAGGTTCTCTTCAAGGGACAA-3', R: 5'-CTCCTGGTATGAAATGGCAAA-3'; POMC F: 5'--3', R: 5'--3'; GAPDH primers were rodent GAPDH control reagents from Applied Biosystems. Measurement of GAPDH RNA levels served as an internal control for all experiments. Amplification was performed for 1 cycle of a sequential incubation at 50 °C for 2 min, 95 °C for 10 min, and subsequent 40 cycles of a consecutive incubation at 94 °C for 15 sec, 60 °C for 30 sec and 72 °C for 35 sec. The individual gene expression value was calculated after normalization to GAPDH.

Plasma analysis of hormones

Plasma was analyzed for corticosterone levels by a competitive ELISA (ImmunoDiagnostic Systems) according to manufacturer's direction. All samples were run on one 96-well plate. Blood used for catecholamine measurement was mixed with EDTA (2 mg/mL) and ascorbic acid (100 μg/mL, Sigma). Plasma was collected and epinephrine and norepinephrine levels were analyzed using Adrenaline & Noradrenaline ELISA assay kit (Eagle Biosciences, Nashua, NH) following manufacturer's recommendations.

Statistics.

Differences in average body weight, tumor volume, tumor number, hormone levels and cytokine levels were assessed using two-way ANOVA followed by Bonferroni post-test. To evaluate tumor type and lung tumor incidence, Chi-square tests were performed.

5.3   Results

β-EP neuronal transplantation into the hypothalamus suppresses mammary tumor growth and metastasis in both normal and fetal alcohol exposed animals.

To make our study more relevant to clinical practice, we used a "detection-and-treat" paradigm instead of "prevention". To be more specific, fetal alcohol exposed or control animals were first injected with a dose of carcinogen, and observed for appearance of tumor. Tumors started to appear as early as 5 weeks after MNU injection, and the occurrence of new tumors in previously tumor-free animals surged at 9~10 weeks after MNU injection. Once the tumor was detected by palpation, the animal was then treated with neuronal transplantation, and followed up by observation of tumor growth. Body weights of these animals in different treatment groups didn't show a significant difference, although there seemed to be a trend for AF+control and PF+control groups to be lower than the other groups (Fig. 13A). This trend may imply growth deficiency in AF+control caused by fetal alcohol exposure. The reduced body weight of animals in PF+control groups may be related to the food restriction during fetal development, because this group was pair-fed the same amount of diet as the AF group. A significant effect of β-EP neuronal transplantation on tumor volume started at 6 weeks after the transplantation, with the tumor volume in all the β-EP -transplanted groups suppressed to a similar low level (Fig. 13B). In animals transplanted with cortical neurons, fetal alcohol exposure significantly increased the tumor volume from 6 weeks to 9 weeks after detection of the tumor compared to AD and PF rats, which is consistent with the previous publication [9]. After 9 weeks, the tumor volume AF animals didn't increase much, primarily because many animals in that group already had too much tumor burden and were sacrificed. β-EP transplantation also suppressed the tumor numbers in all the treated groups (Fig. 13C.).

We have previously shown that β-EP neuron transplants have potent effect on eliminating MADB106 cell metastasis from the lung (Chapter 2). Here we tried to determine the effect of

fetal alcohol exposure on MADB106 cell metastasis, as well as the effect of β-EP neuronal transplantation on inhibition of metastasis in fetal alcohol exposed animals. Examination of the plasma drawn during cell inoculation showed that fetal alcohol exposure significantly increased basal corticosterone level of the rats, while this hyper-activity of stress axis is eliminated by β-EP transplantation (Fig. 13D). Four weeks after tumor cell inoculation, 70~80% of AD and PF animals that had control cell transplantation developed tumor in the lung, while 100% of AF animals developed tumor in the lung (Fig. 13E). β-EP neuronal transplantation significantly reduced the incidence of lung metastasis in all the AF, PF and AD groups (Fig. 13E.), although it didn't completely eliminate the occurrence of tumor in AF and PF groups. Counting of the surface tumor granule numbers revealed the same trend of β-EP transplantation inhibiting the lung retention of tumor cells (Fig. 13F, G.). Animals with β-EP transplants had little or no tumor granule on the lung, while animals with control cell transplants had an average of 20~40 granules on the surface of the lung. AF+control animals had a trend of increased granule number over PF+control animals, but the difference was not statistical significant.

MNU-induced mammary cancer progression
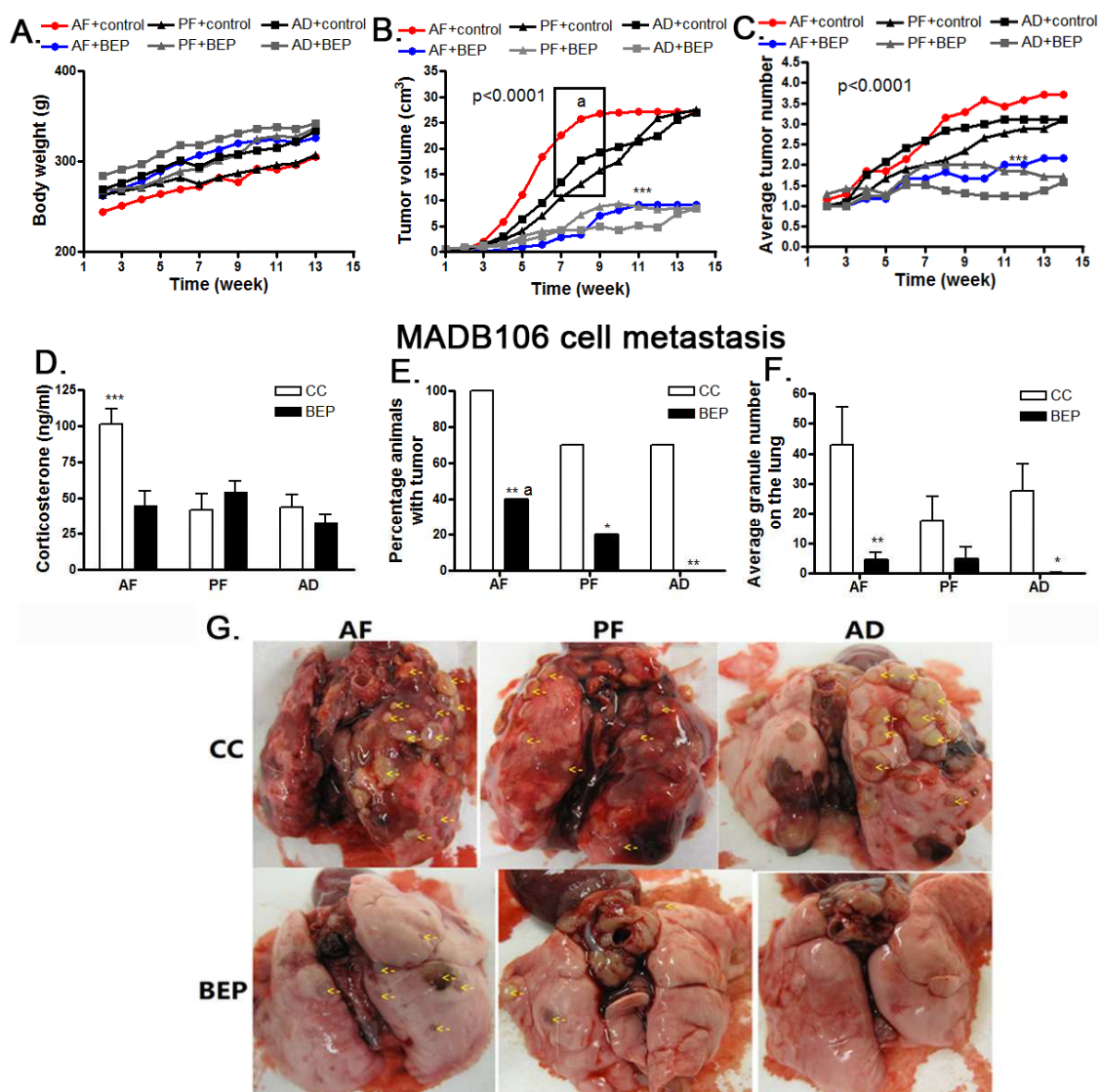
Figure 13: Effect of β-EP neuronal transplant in the hypothalamus on mammary tumor growth and metastasis in both control and fetal alcohol exposed rats.

Animals were exposed to alcohol during fetal period (PD7~21). For the carcinogen-induced mammary cancer model, animals were administered with a single dose of MNU (50 mg/kg body weight) at 49 days of age. After the MNU injection, animals were palpated every week for detection of tumors. Once an animal was found to get tumor, the animal was randomly assigned to transplantation of either cortical neurons or β-EP neurons. After the cell transplantation, animals were palpated every week until 14 weeks after tumor detection, or until the tumor exceeded 3 cm in one dimension. A. Body weight of animals. B. Average tumor volume per animal in each group. C. Average tumor number per animal in each group. For the mammary cancer metastasis study, rats were transplanted with β-EP cells or cortical cells at young adult age. After recovery, they were then inoculated with MADB106 mammary carcinoma cells via jugular vein, and sacrificed after 4 weeks. D. Corticosterone levels in plasma collected at time of tumor inoculation. E.

Percentage of animals with tumor in the lung in each group. F. Average tumor numbers in the lung of each animal in each group. Data in panels A~E were analyzed using 2-way ANOVA followed by Bonferroni posttest and in B and C, all groups with β-EP were significantly different from all the groups with control, p<0.0001. In panel B, AF+control was significantly different from AD control, as indicated by a. In panel D, AD+control was significantly different from all other groups, p<0.0001. F was analyzed using Chi-square test. *: p<0.05, **: p<0.01, comparing to the control counterparts. a: p<0.05 comparing to AD+ β-EP group.

Injection of cAMP-delivering nanospheres inhibited the higher incidence of mammary cancer in fetal alcohol exposed rats

We tested the effect of cAMP-delivering nanospheres on the stress hormones, immune function and mammary cancer growth in fetal alcohol exposed female rats. After injection of MNU which induces mammary cancer growth, we palpated the rats every week, and only took rats that had detectable tumors for injection of nanospheres. Tumor growth was then observed every week by palpation until 10 weeks after the first detection of tumor, when animals were sacrificed and tissues were collected. We found that injection of cAMP-delivering nanospheres significantly decreased tumor size (Fig. 14A) and number of tumors (Fig. 14B) in both control and fetal alcohol exposed animals. By histopathological analysis, we found that injection of cAMP-delivering nanospheres decreased the rate of malignant tumors, which were increased in the case of fetal alcohol exposed animals (Fig. 14C). Moreover, nanosphere injection even cleared out some already initiated tumors in AD and PF groups (Fig. 4C). Examination of plasma hormone level showed that fetal alcohol exposed rats had increased levels of epinephrine and norepinephrine, while injection of cAMP-delivering nanosphere decreased the levels of epinephrine and norepinephrine back to normal (Fig. 14H, I). Measurement of splenic cytokines showed that IFN-γ (Fig. 14D) and granzyme B (Fig. 14E) which are essential cytokines for natural killer (NK) cell function against cancer, are decreased in fetal alcohol exposed animals, and increased in cAMP-delivering nanosphere injected animals. The level of perforin wasn't changed (Fig. 14F). The level of pro-inflammatory cytokine TNF-α was increased in fetal alcohol

exposed animals, but inhibited by injection of cAMP-delivering nanospheres (Fig. 14G). These data suggest injection of the cAMP-delivering nanospheres inhibited carcinogen induced mammary cancer growth in both normal and fetal alcohol exposed animals, possibly by deceasing stress and increasing innate immune function.
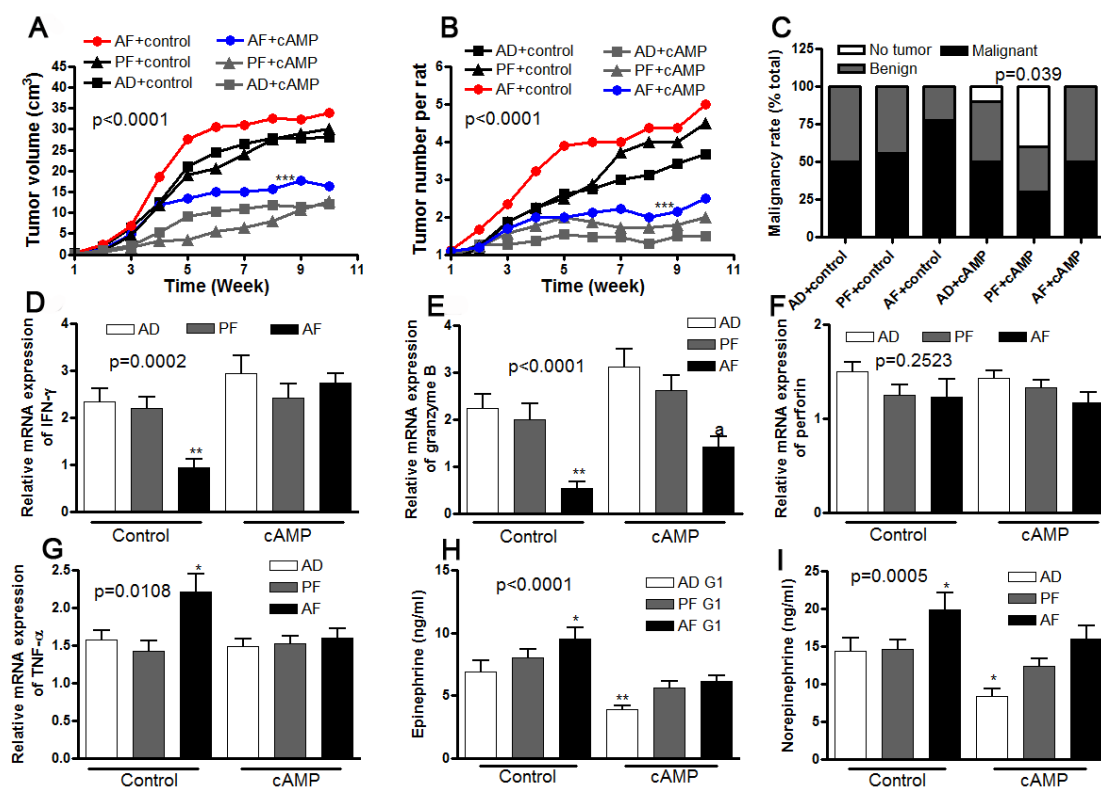
Figure 14: Effect of dbcAMP-delivering nanospheres injection on mammary cancer growth, spleen cytokine levels and plasma catecholamine levels.

Fetal alcohol exposed animals were injected with a single dose of MNU at the age of 49 days old, and were palpated every week for detection of mammary tumor. Once an animal was found to have tumor, it was randomly assigned to receive injection of control nanospheres or nanospheres containing dbcAMP. Afterwards, these animals were continued to be palpated every week until 10 weeks after tumor detection, or until one dimension of the tumor reachs 3 cm. A. Average volume of tumors on each animal in each treatment group. B. Average tumor number on each animal in each group. C. Percentage of each histological tumor type that developed in each grou. D. IFN-γ expression in the spleen. E. Granzyme B expression in the spleen. F. Perforin expression in the spleen. G. TNF-α expression in the spleen. H. Epinephrine concentration in the plasma. I. Norepinephrine concentration in the plasma. Data in panels A, B and D~I were analyzed using two-way ANOVA, followed by Bonferroni posttest. In A and B, all the groups with cAMP were significantly different from all the groups with control. But there wasn't difference between groups with cAMP or between groups with control. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$,

comparing to AD+control group. a: p<0.05 comparing to AD+cAMP group. C was analyzed using Chi-square test.

5.4   Discussion

The increased incidence of tumor origenesis in fetal alcohol exposed offspring may be due to multiple factors, including: 1) defects in immune function, which leads to impaired immune surveillance; 2) abnormal hormone levels, such as stress hormones, which suppress immune function; and 3) altered organ development and gene expression in the affected tissue by developmental imprinting. Our lab has focused on the effect of FAE on neuroendocrine regulation of the immune function. Fetal alcohol exposure causes apoptotic death of neurons in the central nervous system, including the β-EP neurons [42-43]. Lower numbers of β-EP neurons or peptide production have been also found in brains of patients with schizophrenia, depression, and obese patients [20-22], and these pathological conditions were correlated with higher incidences of cancers and infections [23-25]. β-EP, as an endogenous opioid polypeptide compound, is produced by the pituitary gland and the hypothalamus in vertebrates during exercise, excitement, pain, consumption of spicy food and orgasm, and it resembles the opiates in its abilities to produce analgesia and a feeling of well-being [18, 220]. Central administration of β-EP was shown to increase immune function, such as NK cytolytic activity and lymphocyte proliferative response to mitogens [146-147, 251].

The data presented here show that by increasing the β-EP neuronal number and peptide production in the hypothalamus, either by transplantation of *in vitro* produced β-EP neurons or by injection of a reagent that promotes NSCs to differentiate into β-EP neurons, the hyper-active stress axis was suppressed and mammary cancer growth and metastasis was inhibited in fetal alcohol exposed animals. These effects may possibly be due to the impact of β-EP on the suppression of the HPA axis and the sympathetic nervous system, and regulation of cytokine

levels, such as increasing IFN-γ and granzyme B levels, and decreasing TNF-α levels. Our study identified the possible role of β-EP neuronal deficiency in the abnormal stress and immune function, as well as increased incidence of cancer in fetal alcohol exposed animals. Furthermore, procedures that enhance β-EP production in the hypothalamus could suppress tumor growth in AF animals to a level that is lower than AD animals with cortical cell transplantation, indicating that the amount of β-EP increased by the treatments not only replenished the loss of β-EP neurons caused by FAE, but also supplemented additional β-EP neurons to these animals. Normal animals could also benefit from the increased amount of β-EP neurons in the hypothalamus and gain resistance to mammary tumor growth and metastasis. Therefore these data suggest a potential therapeutic method for treatment of stress, immune or neoplastic problems in patients with FASD.

CHAPTER 5

6.   Chapter 5: Evidence for possible mediation of innate immune system in effect of β-EP neuronal transplantation on cancer growth.

6.1   Introduction

A little stress may be good for the organism to adapt to the environment. However, prolonged stress, or stress that is over a threshold, may cause a number of health problems, such as mental problems, autoimmune diseases, cardiovascular diseases, and different types of cancer. Researches using animal models have shown that physical or psychological stressors reduce immune functions against cancer and increase cancer development [269, 342]. β-endorphin (β-EP), an endogenous opioid polypeptide produced by neurons and immune cells, is known to have the ability to inhibit stress hormone production, produce analgesia and a feeling of well-being [18-19]. β-EP neurons in the arcuate nucleus of the hypothalamus innervate the paraventricular nucleus (PVN), and inhibit the activity of both HPA axis and SNS. We have previously shown that by transplantation of *in vitro* produced β-EP neurons into the PVN, we could inhibit the activity of the stress axis, promote innate immune function, as well as inhibit the development of different types of cancers [113, 221, 336]. However, the mechanism by which the β-EP neuronal transplants inhibit cancer growth was not clear. Here we used the athymic hairless nude rat to tease apart the effect from different immune components. These nude rats have a complete loss of thymus, and therefore lack the population of T cells. But non-thymus dependent immune activities, such as cytotoxicity by NK cells or macrophages, and IgM-antibody production to T-independent antigens, are intact [343]. Another benefit of using nude rats is that their immune deficiency allows subcutaneous growth of different tumor cell lines, and therefore is good for the direct detection and observation of tumor development. We used this rat model to check the effect of β-EP neuron transplants on the subcutaneous growth of a rat mammary carcinoma cell line.

6.2   Materials and Methods

Animals

Female RNU rats (NIH nude rats) were purchased from Charles River (Wilmington, MA) and housed under pathogen-free conditions. At 2 months old, animals were anesthetized with sodium pentobarbital (50 mg/kg body weight, Sigma Aldrich, St. Louis, MO) and injected with 20,000 cortical neurons (control) or β-EP neurons in both sides of PVN of the hypothalamus using stereotactic procedures (the coordinates were set 1.8 mm behind bregma, 0.5 mm lateral, and 7.5 mm below the cortex). MADB106 mammary carcinoma cell line was maintained in RPMI1640+10 % FBS+1 % antibiotics (Sigma), and trypsinized for subculture or injection. After recovery from the surgery, these animals were injected with MADB106 cells (100,000/0.2 mL/rat) on the right flank, and the tumor growth was monitored and the size measured with a caliper every other day. After 2 weeks, the animals were sacrificed, and tumor colonization on the lung was counted.

β-EP cells preparation and transplantation

Same as described in Chapter 2.

Statistics

Difference in tumor volume was assessed using two-way ANOVA followed by Bonferroni post-test. To evaluate incidences of tumor under the arm and lung metastasis, Chi-square tests were performed. A $p<0.05$ was considered significant.

6.3   Results

The rat mammary cancer cell line MADB106 cells were injected under the skin on the right flank of the rats. Within several days, the injected cells developed into a palpable clump at the site of

cell injection. In about half of the animals injected with control neurons, we observed the growth of another tumor underneath the arm at the same side of the original tumor (Fig 15. A), indicating a metastasis ability of the MADB106 tumor cells. Since the armpit is rich in lymph nodes, which filter the cells carried by lymphatic vessels, it is highly possible that these observed metastases to the armpits were carried out by the lymphatic system. However, this kind of secondary tumor didn't occur in any of the β-EP neuronal transplanted animals (Fig. 15C). After sacrifice of the animals, we checked for possible metastasis to the inner organs, and found tumor granules located in the lungs of most of the control animals (9 out of 10), but only 2 of the β-EP transplanted animals (Fig. 15A, D). Measurement of the original tumor growth also showed that β-EP transplantation slightly decreased the tumor growth compared to control animals (Fig. 15B).
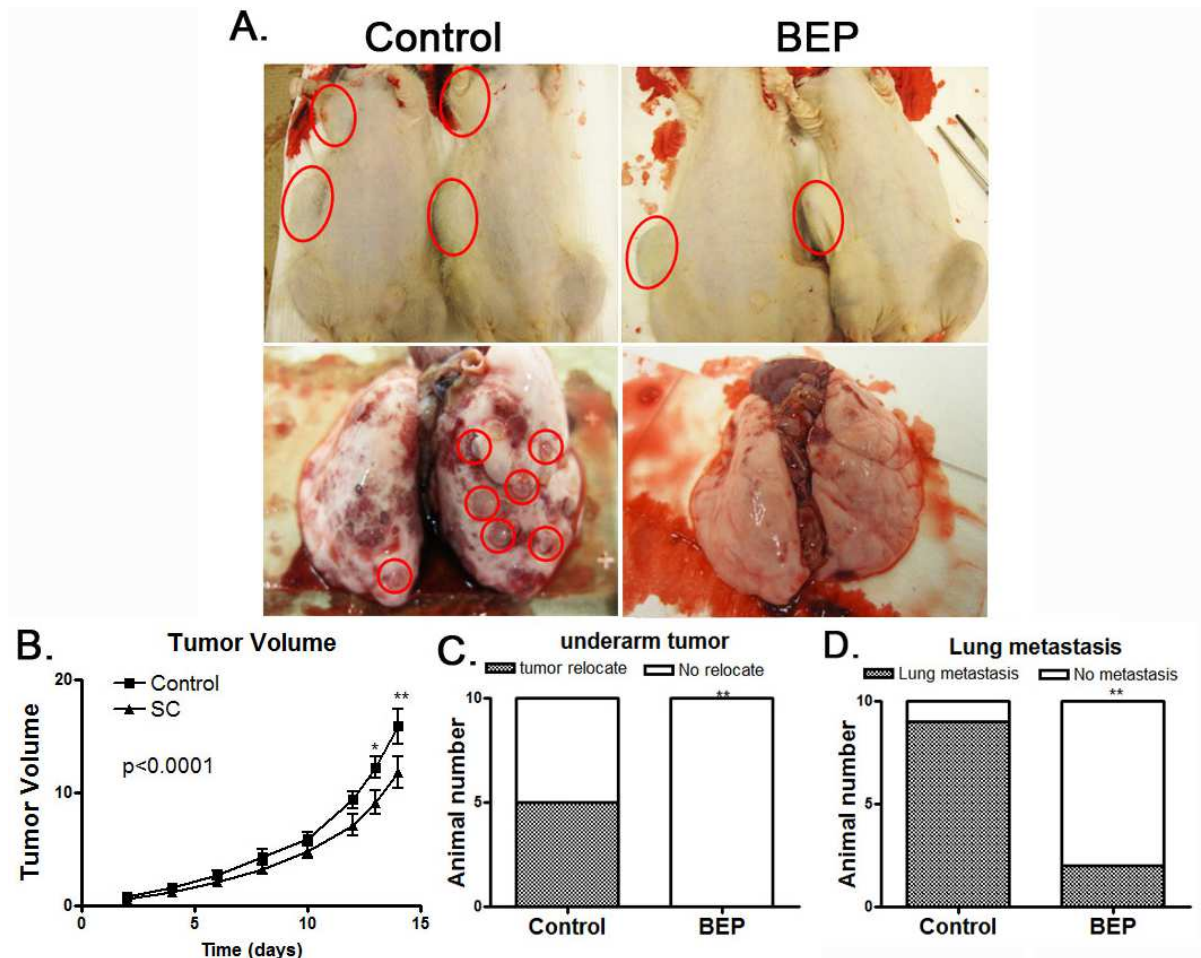
Figure 15: Effect of β-EP neuronal transplant on MADB106 mammary carcinoma tumor growth and metastasis in T-cell deficient nude rats.

Animals were transplanted with β-EP neurons or cortical neurons as control into PVN bilaterally, and then injected with MADB106 mammary tumor cells subcutaneously on the right flank. Tumor size was measured every other day by palpation after the injection. A. Representative photos showing tumor growing that the site of injection in both β-EP and control animals. Additionally, control animals showed tumor growth underneath the arm on the same side where the original tumor cells were administered and they also had tumor metastasis to the lungs. B. Graph showing the volume of original tumor at the site of injection. Metastasis wasn't found in other internal sites or organs in addition to the lungs. C. Graph represents number of rats with tumor appearing underneath the arm (n=10 in both groups). D. Graph represents number of rats had tumor metastasis in the lung (n=10). Data of B were analyzed using 2-way ANOVA followed by Bonferroni posttest. Data of C and D were analyzed using Chi-square test. *: p<0.05, **: p<0.01.

**6.4	Discussion**

The nude rats used in this experiment have a congenital phenotype of loss of thymus, which is the site for T cell maturation. Therefore they don't have functional T cells, which is important for cell-mediated adaptive immune function. The B-lymphocyte branch of the immune system is intact, insofar as it can function without T-cell assistance. $CD4^+$ T helper cells play a role in maturation of B cells into plasma cells, which is essential for the humoral immunity. According to our data, the inhibition of metastasis in β-EP transplanted animals therefore is not related to the T cell-related function, largely the adaptive immunity. Since the MADB106 cell line is known to be sensitive to NK cell activity [312], it is highly possible that the increased immune function in β-EP transplanted rats were mediated through the innate immune system, especially NK cell function.

We have previously tried injection of the same cell line subcutaneously into normal Fischer and Sprague Dawley rats, but no tumor growth could be achieved. However, this cell line grew into a tumor in the athymic nude rats. This result indicates that T cell-related functions are necessary for inhibition of the subcutaneous growth of the MADB106 tumor cell line. Innate immune function may also be involved in this inhibition of cell graft growth, since the β-EP transplanted animals which had higher innate immune activities showed a little slower growth of the original tumor. However, innate immune activity is not sufficient for this inhibition, because there was still growth of tumors under the skin.

Stress differently regulates immune cell activities at different immune compartments. In the blood, stress induces reduction of lymphocyte number, and this reduction can be eliminated by adrenalectomy or steroidogenesis inhibition [344]. The reduction of NK cells adhering to the lung endothelium induced by surgical stress can also be eliminated by COX-2 and β-adrenergic blocker [344]. However, the stress-induced reduction of leukocytes in the skin cannot be

prevented by either adrenalectomy or β-adrenergic or glucocorticoid antagonists [345]. Additionally, administration of corticosterone or epinephrine didn't impact leukocyte numbers in the skin, but did affect leukocyte numbers in the blood [345]. These facts indicate that immune function in the skin is regulated in a way other than the HPA axis and SNS. In our case, it seems that the β-EP transplants were not able to eliminate the cells growing under the skin, but were quite potent in inhibiting the metastasis, which is mediated through the circulatory system. This finding is in accordance with our previous hypothesis that these β-EP transplants inhibit tumor growth through regulation of the stress response of the HPA axis and the ANS.

In conclusion, by using the athymic nude rats as a model, we showed that β-EP transplants inhibit mammary cancer cell metastasis and growth in a T-cell independent manner.

SUMMARY & CONCLUSIONS

7   Summary and Conclusions

From this dissertation work, three major conclusions can be generated: 1) Transplantation of β-EP neurons and injection of dbcAMP-delivering nanospheres can both increase the hypothalamic β-EP peptide production, inhibit the levels of stress hormones, increase immune functions, and inhibit mammary cancer progression and metastasis. 2) Measures that increase β-EP production in the hypothalamus could reverse the effect of fetal alcohol exposure on stress axis, immune function and susceptibility to mammary cancer progression and metastasis. 3) The effect of activated β-EP neurons on cancer growth may be mediated through activation of the PSNS and inhibition of the SNS, which then activate the innate immune activity such as NK cytolytic activity and macrophage activity.

The idea that physical or psychological stress has important implications for human mental and physical wellbeing has long been proposed. This idea is supported by many clinical and animal researches by showing that chronic stress conditions such as post-traumatic stress disorders and depress could cause or exacerbate different somatic diseases including cardiovascular diseases, diabetes, gastrointestinal disorders and cancer, primarily through immune-mediated mechanisms [236, 346]. On the other hand, measures that inhibits stress reactions such as β-adrenergic receptor blockers have been used to promote health, such as to control or prevent heart failure [347] and wasting in cancer patients [348]. However, whether inhibiting the stress activity could promote immune function and inhibit cancer growth is not clear, possibly due to the difficulty to find a way to persistently suppress stress.

Opioids are a class of psychoactive chemicals that give analgesic and euphoric feeling when consumed. They all bind to opioid receptors which exist in the periphery and the CNS and are known to inhibit production of CRH, therefore inhibiting the activity of the stress axis. β-EP is an important type of endogenous opioid originally extracted from the camel pituitary, and was found

to be highly potent on suppression of pain perception, either administered to the CNS or periphery [349-350]. Our research utilized the anxiolytic property of β-EP and created a steady supply of endogenous β-EP by either transplanting β-EP neurons or induction of differentiation of NSCs in the hypothalamus. For the first time, we demonstrated that by increasing the production of β-EP peptide in the hypothalamus, we could promote the function of innate immunity and inhibit mammary cancer development and metastasis. This finding supports the idea that stress affects somatic health by showing alleviating stress promotes health and suppresses the development of diseases, and therefore indicating a novel therapeutic method for treating immune suppression and neoplastic diseases.

Reduced β-EP neuronal number, increased tone of stress axis and suppressed immune functions are all interrelated with each other [14, 222, 250]. Each of these three symptoms can be observed in fetal alcohol exposed patients and/or lab animals [6-7, 91, 111, 139]. However, whether there were any causal effects between these three aspects was not clear. In our experiment, we supplemented β-EP neurons in the hypothalamus by either directly transplanting *in vitro*-generated β-EP neurons into the hypothalamus, or inducing *in vivo* β-EP neuronal differentiation. By doing so, we could suppress the hyper-active stress axis in fetal alcohol exposed animals, and increase their immune function. These results indicate that the modification of β-EP neurons is at the upstream of these serial changes of stress activity and immune function that occur in fetal alcohol exposed patients, as is illustrated by the following graph (Fig. 16). These results provide direct evidence that loss of β-EP caused by fetal alcohol exposure leads to hyper-active stress axis and suppressed immune function, indicating a possible therapy for treating stress and immune problems in fetal alcohol exposed patients.
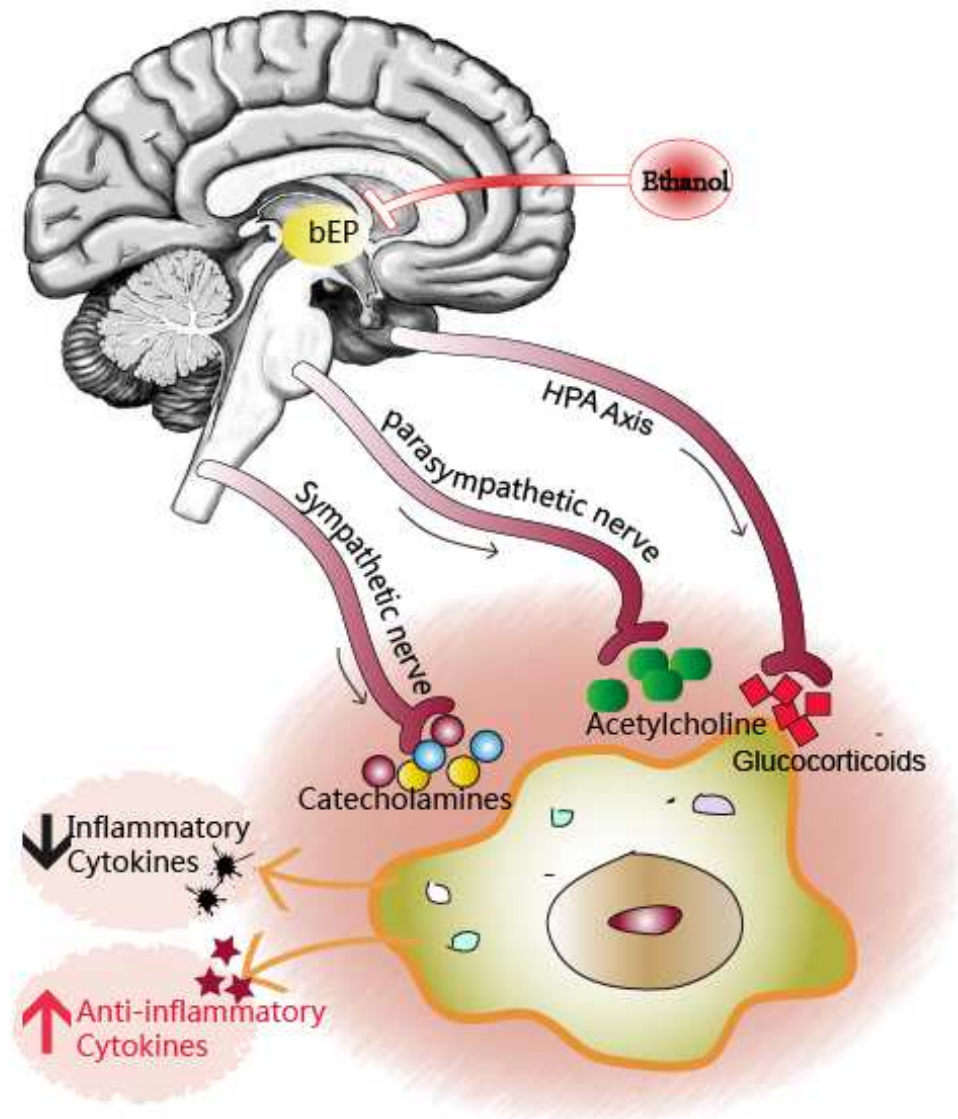
Figure 16: Proposed model of effect of fetal alcohol exposure on stress and immune function.

Adapted from Sarkar 2011 [336].

Fetal alcohol exposure causes reduction of β-EP neuronal numbers and POMC expression in the hypothalamus, which results in decreased feedback regulation of the HPA axis and SNS. The hyper-activation of the HPA axis and SNS, as well as hypo-activation of PSNS cause increased release of glucocorticoids and catecholamines, which directly inhibit the activity of immune cells. Therefore these immune cells produce an increased amount of pro-inflammatory cytokines, which increase cancer progression, and a decreased amount of anti-inflammatory cytokines.

In addition to stress and immune problems, fetal alcohol exposure is also known to cause increased susceptibilities to different types of cancers. We have also observed that in fetal alcohol exposed animals, there is an increased incidence of malignant tumor when treated with carcinogen that could induce both benign and malignant tumors. Malignancy is defined by the ability of tumor cells to metastasize and invade into other parts of the body. The conversion from non-metastatic tumor to metastatic tumor is achieved by a transformation of the cells called endothelial-mesenchymal transition (EMT). By testing the existence of EMT markers in tumor samples, we showed that transplantation of β-EP neurons significantly reduces the incidence of EMT in carcinogen-induced tumors (Fig. 5), and the hypothesized mechanism is as follow (Fig. 17). Tumor cells underwent EMT acquire more property as cancer cells and loss the identifying marker for self-recognition, therefore can be detected and destroyed by surveillance of immune system. Since increased β-EP production in the hypothalamus can affect the activity of the ANS, it can then promote immune function, regulate the cytokine production, promote apoptosis of abnormal cells, and finally inhibit EMT of tumor tissue.
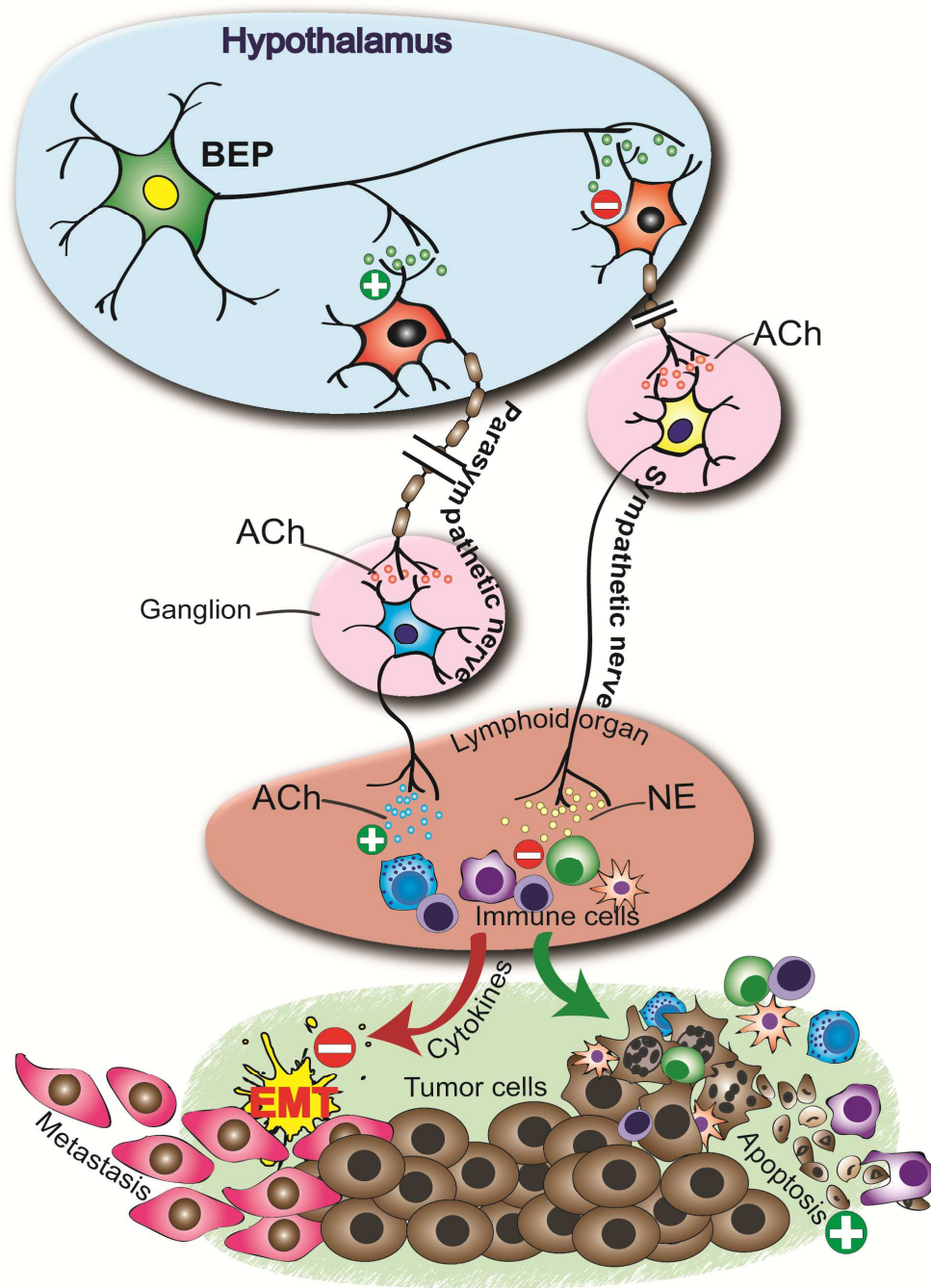
Figure 17: Proposed mechanism of effect of hypothalamic β-EP peptide on the inhibition of cancer progression

β-EP produced in the hypothalamus inhibits the SNS, and in turn activates the PSNS. This causes increased release of acetylcholine (ACh), and decreased release of catecholamines such as norepinephrine (NE) into their innervated peripheral organs, including the spleen. In the spleen, the decreased level of NE and increased ACh activates immune cells, and on one hand cause increased cytosis and apoptosis of tumor cells, and on the other hand produce cytokines that inhibit the EMT.

Connecting all the parts together, the proposed outline of the regulatory pathway is shown by the figure as follows (Fig. 18): Fetal alcohol exposure inhibits the production of β-EP peptide in the hypothalamus, which then causes increased activity of the stress axis, and therefore inhibits immune function and increases cancer incidence and progression. Conversely, by increasing the hypothalamic β-EP production using β-EP neuronal transplantation or injection of dbcAMP-delivering nanospheres, we could inhibit the stress axis activity, therefore increasing immune function, and finally inhibit cancer development in fetal alcohol exposed animals.
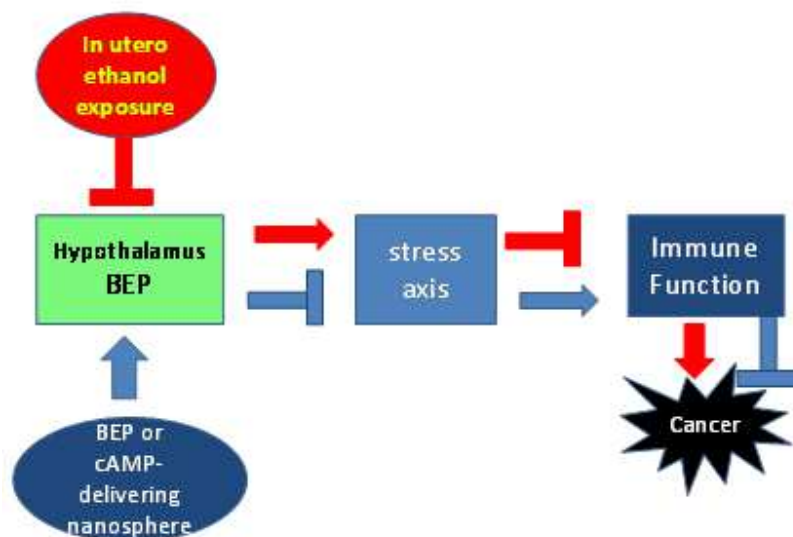
Figure 18: Proposed mechanism pathway of β-EP control of stress and cancer in fetal alcohol exposed animals.

8.   Literature Cited

1.   Kelly, S.J., et al., *Animal models of fetal alcohol spectrum disorders: impact of the social environment.* Dev Disabil Res Rev, 2009. **15**(3): p. 200-8.

2.   O'Leary, C.M., *Fetal alcohol syndrome: diagnosis, epidemiology, and developmental outcomes.* J Paediatr Child Health, 2004. **40**(1-2): p. 2-7.

3.   Sampson, P.D., et al., *Incidence of fetal alcohol syndrome and prevalence of alcohol-related neurodevelopmental disorder.* Teratology, 1997. **56**(5): p. 317-26.

4.   Green, J.H., *Fetal Alcohol Spectrum Disorders: understanding the effects of prenatal alcohol exposure and supporting students.* J Sch Health, 2007. **77**(3): p. 103-8.

5.   Jacobson, S.W., et al., *Effects of prenatal alcohol and cocaine exposure on infant cortisol levels.* Dev Psychopathol, 1999. **11**(2): p. 195-208.

6.   Hellemans, K.G., et al., *Prenatal alcohol exposure: fetal programming and later life vulnerability to stress, depression and anxiety disorders.* Neurosci Biobehav Rev, 2010. **34**(6): p. 791-807.

7.   Chiappelli, F. and Taylor, A.N., *The fetal alcohol syndrome and fetal alcohol effects on immune competence.* Alcohol Alcohol, 1995. **30**(2): p. 259-62.

8.   Johnson, S., et al., *Immune deficiency in fetal alcohol syndrome.* Pediatr Res, 1981. **15**(6): p. 908-11.

9.   Polanco, T.A., et al., *Fetal alcohol exposure increases mammary tumor susceptibility and alters tumor phenotype in rats.* Alcohol Clin Exp Res, 2010. **34**(11): p. 1879-87.

10.   Hilakivi-Clarke, L., et al., *In utero alcohol exposure increases mammary tumorigenesis in rats.* Br J Cancer, 2004. **90**(11): p. 2225-31.

11.   Severson, R.K., et al., *Cigarette smoking and alcohol consumption by parents of children with acute myeloid leukemia: an analysis within morphological subgroups--a report from the Childrens Cancer Group.* Cancer Epidemiol Biomarkers Prev, 1993. **2**(5): p. 433-9.

12.   Mongraw-Chaffin, M.L., et al., *Maternal smoking, alcohol, and coffee use during pregnancy and son's risk of testicular cancer.* Alcohol, 2009. **43**(3): p. 241-5.

13.   Zhang, X., et al., *Prenatal alcohol exposure and fetal programming: effects on neuroendocrine and immune function.* Exp Biol Med (Maywood), 2005. **230**(6): p. 376-88.

14.   Webster Marketon, J.I. and Glaser, R., *Stress hormones and immune function.* Cell Immunol, 2008. **252**(1-2): p. 16-26.

15.   Smyth, M.J., et al., *Cytokines in cancer immunity and immunotherapy.* Immunol Rev, 2004. **202**: p. 275-93.

16.   Herberman, R.B., *Possible role of natural killer cells and other effector cells in immune surveillance against cancer.* J Invest Dermatol, 1984. **83**(1 Suppl): p. 137s-140s.

17.   Saez Mdel, C., et al., *Exercise-induced stress enhances mammary tumor growth in rats: beneficial effect of the hormone melatonin.* Mol Cell Biochem, 2007. **294**(1-2): p. 19-24.

18.   Akil, H., et al., *Endogenous opioids: biology and function.* Annu Rev Neurosci, 1984. **7**: p. 223-55.

19.   Yermal, S.J., et al., *Perioperative pain, psychological distress, and immune function in men undergoing prostatectomy for cancer of the prostate.* Biol Res Nurs, 2010. **11**(4): p. 351-62.

20.   Kuhn, P. and Sarkar, D.K., *Ethanol induces apoptotic death of beta-endorphin neurons in the rat hypothalamus by a TGF-beta 1-dependent mechanism.* Alcohol Clin Exp Res, 2008. **32**(4): p. 706-14.

21.   Bernstein, H.G., et al., *Fewer beta-endorphin expressing arcuate nucleus neurons and reduced beta-endorphinergic innervation of paraventricular neurons in schizophrenics*

and patients with depression. Cell Mol Biol (Noisy-le-grand), 2002. **48 Online Pub**: p. OL259-65.

22. Pankov Iu, A., et al., *[Screening of mutations in genes of pro-opiomelanocortin in patients with constitutional exogenous obesity].* Vopr Med Khim, 2002. **48**(1): p. 121-30.

23. Irwin, M.R. and Miller, A.H., *Depressive disorders and immunity: 20 years of progress and discovery.* Brain Behav Immun, 2007. **21**(4): p. 374-83.

24. Giovannucci, E. and Michaud, D., *The role of obesity and related metabolic disturbances in cancers of the colon, prostate, and pancreas.* Gastroenterology, 2007. **132**(6): p. 2208-25.

25. Polanco, T.A., et al., *Fetal Alcohol Exposure Increases Mammary Tumor Susceptibility and Alters Tumor Phenotype in Rats.* Alcohol Clin Exp Res.

26. Jones, K.L., et al., *Pattern of malformation in offspring of chronic alcoholic mothers.* Lancet, 1973. **1**(7815): p. 1267-71.

27. Sokol, R.J., et al., *Fetal Alcohol Spectrum Disorder.* JAMA, 2003. **2909**: p. 2996-2999.

28. *Alcohol use and binge drinking among women of childbearing age--United States, 2006-2010.* MMWR Morb Mortal Wkly Rep, 2012. **61**(28): p. 534-8.

29. Floyd, R.L., et al., *Prevention of fetal alcohol spectrum disorders.* Dev Disabil Res Rev, 2009. **15**(3): p. 193-9.

30. Floyd, R.L., et al., *Alcohol use prior to pregnancy recognition.* Am J Prev Med, 1999. **17**(2): p. 101-7.

31. Sokol, R.J., et al., *Fetal alcohol spectrum disorder.* JAMA, 2003. **290**(22): p. 2996-9.

32. Pruett, D., et al., *Fetal alcohol exposure: consequences, diagnosis, and treatment.* Obstet Gynecol Surv, 2013. **68**(1): p. 62-9.

33. Clarren, S.K. and Smith, D.W., *The fetal alcohol syndrome.* N Engl J Med, 1978. **298**(19): p. 1063-7.

34. Clarren, S.K., et al., *Brain malformations related to prenatal exposure to ethanol.* J Pediatr, 1978. **92**(1): p. 64-7.

35. Archibald, S.L., et al., *Brain dysmorphology in individuals with severe prenatal alcohol exposure.* Dev Med Child Neurol, 2001. **43**(3): p. 148-54.

36. Lebel, C., et al., *Imaging the impact of prenatal alcohol exposure on the structure of the developing human brain.* Neuropsychol Rev, 2011. **21**(2): p. 102-18.

37. Streissguth, A.P., et al., *Risk factors for adverse life outcomes in fetal alcohol syndrome and fetal alcohol effects.* J Dev Behav Pediatr, 2004. **25**(4): p. 228-38.

38. O'Connor, M.J., et al., *Psychiatric illness in a clinical sample of children with prenatal alcohol exposure.* Am J Drug Alcohol Abuse, 2002. **28**(4): p. 743-54.

39. Charness, M.E., et al., *Ethanol inhibits neural cell-cell adhesion.* J Biol Chem, 1994. **269**(12): p. 9304-9.

40. Minana, R., et al., *Alcohol exposure alters the expression pattern of neural cell adhesion molecules during brain development.* J Neurochem, 2000. **75**(3): p. 954-64.

41. Greenberg, D.A., *Linking acquired neurodevelopmental disorders to defects in cell adhesion.* Proc Natl Acad Sci U S A, 2003. **100**(14): p. 8043-4.

42. De, A., et al., *Cyclic AMP and ethanol interact to control apoptosis and differentiation in hypothalamic beta-endorphin neurons.* J Biol Chem, 1994. **269**(43): p. 26697-705.

43. Chen, C.P., et al., *Ethanol induces apoptotic death of developing beta-endorphin neurons via suppression of cyclic adenosine monophosphate production and activation of transforming growth factor-beta1-linked apoptotic signaling.* Mol Pharmacol, 2006. **69**(3): p. 706-17.

44.   Ramachandran, V., et al., *Ethanol-induced oxidative stress precedes mitochondrially mediated apoptotic death of cultured fetal cortical neurons.* J Neurosci Res, 2003. **74**(4): p. 577-88.

45.   Ramachandran, V., et al., *In utero ethanol exposure causes mitochondrial dysfunction, which can result in apoptotic cell death in fetal brain: a potential role for 4-hydroxynonenal.* Alcohol Clin Exp Res, 2001. **25**(6): p. 862-71.

46.   Kotch, L.E., et al., *Ethanol-induced teratogenesis: free radical damage as a possible mechanism.* Teratology, 1995. **52**(3): p. 128-36.

47.   de La Monte, S.M. and Wands, J.R., *Mitochondrial dna damage and impaired mitochondrial function contribute to apoptosis of insulin-stimulated ethanol-exposed neuronal cells.* Alcohol Clin Exp Res, 2001. **25**(6): p. 898-906.

48.   Seiler, A.E., et al., *Differential effects of ethanol on insulin-like growth factor-I receptor signaling.* Alcohol Clin Exp Res, 2000. **24**(2): p. 140-8.

49.   Cartwright, M.M. and Smith, S.M., *Increased cell death and reduced neural crest cell numbers in ethanol-exposed embryos: partial basis for the fetal alcohol syndrome phenotype.* Alcohol Clin Exp Res, 1995. **19**(2): p. 378-86.

50.   Sari, Y. and Zhou, F.C., *Prenatal alcohol exposure causes long-term serotonin neuron deficit in mice.* Alcohol Clin Exp Res, 2004. **28**(6): p. 941-8.

51.   Schenker, S., et al., *The transfer of cocaine and its metabolites across the term human placenta.* Clin Pharmacol Ther, 1993. **53**(3): p. 329-39.

52.   Schenker, S., et al., *Human placental vitamin B6 (pyridoxal) transport: normal characteristics and effects of ethanol.* Am J Physiol, 1992. **262**(6 Pt 2): p. R966-74.

53.   Kay, H.H., et al., *Ethanol exposure induces oxidative stress and impairs nitric oxide availability in the human placental villi: a possible mechanism of toxicity.* Am J Obstet Gynecol, 2000. **182**(3): p. 682-8.

54.   Taylor, S.M., et al., *Pressor effect of ethanol in the isolated perfused human placental lobule.* Eur J Pharmacol, 1994. **270**(4): p. 371-4.

55.   Padmanabhan, R., *Histological and histochemical changes of the placenta in fetal alcohol syndrome due to maternal administration of acute doses of ethanol in the mouse.* Drug Alcohol Depend, 1985. **16**(3): p. 229-39.

56.   Riley, E.P. and McGee, C.L., *Fetal alcohol spectrum disorders: an overview with emphasis on changes in brain and behavior.* Exp Biol Med (Maywood), 2005. **230**(6): p. 357-65.

57.   Guerri, C., et al., *Foetal Alcohol Spectrum Disorders and alterations in brain and behaviour.* Alcohol Alcohol, 2009. **44**(2): p. 108-14.

58.   Svinarich, D.M., et al., *Ethanol-induced expression of cytokines in a first-trimester trophoblast cell line.* Am J Obstet Gynecol, 1998. **179**(2): p. 470-5.

59.   McLeod, W., et al., *Effect of maternal ethanol ingestion on fetal breathing movements, gross body movements, and heart rate at 37 to 40 weeks' gestational age.* Am J Obstet Gynecol, 1983. **145**(2): p. 251-7.

60.   Garro, A.J., et al., *Ethanol consumption inhibits fetal DNA methylation in mice: implications for the fetal alcohol syndrome.* Alcohol Clin Exp Res, 1991. **15**(3): p. 395-8.

61.   Haycock, P.C. and Ramsay, M., *Exposure of mouse embryos to ethanol during preimplantation development: effect on DNA methylation in the h19 imprinting control region.* Biol Reprod, 2009. **81**(4): p. 618-27.

62.   Haycock, P.C., *Fetal alcohol spectrum disorders: the epigenetic perspective.* Biol Reprod, 2009. **81**(4): p. 607-17.

63.   Wang, X., et al., *Acute alcohol exposure induces apoptosis and increases histone H3K9/18 acetylation in the mid-gestation mouse lung.* Reprod Sci, 2010. **17**(4): p. 384-90.

64. Miranda, R.C., et al., *MicroRNAs: master regulators of ethanol abuse and toxicity?* Alcohol Clin Exp Res, 2010. **34**(4): p. 575-87.

65. Feng, J., et al., *Epigenetic regulation of neural gene expression and neuronal function.* Pediatr Res, 2007. **61**(5 Pt 2): p. 58R-63R.

66. Wilkemeyer, M.F., et al., *Antagonists of alcohol inhibition of cell adhesion.* Proc Natl Acad Sci U S A, 2000. **97**(7): p. 3690-5.

67. Peng, Y., et al., *Ascorbic acid inhibits ROS production, NF-kappa B activation and prevents ethanol-induced growth retardation and microencephaly.* Neuropharmacology, 2005. **48**(3): p. 426-34.

68. Heaton, M.B., et al., *Amelioration of ethanol-induced neurotoxicity in the neonatal rat central nervous system by antioxidant therapy.* Alcohol Clin Exp Res, 2000. **24**(4): p. 512-8.

69. Long, L., et al., *The preventive effect of oral EGCG in a fetal alcohol spectrum disorder mouse model.* Alcohol Clin Exp Res, 2010. **34**(11): p. 1929-36.

70. Ballard, M.S., et al., *Vitamin A, folate, and choline as a possible preventive intervention to fetal alcohol syndrome.* Med Hypotheses, 2012. **78**(4): p. 489-93.

71. Kim, J.A., et al., *Effects of maternal ethanol consumption and buspirone treatment on 5-HT1A and 5-HT2A receptors in offspring.* Alcohol Clin Exp Res, 1997. **21**(7): p. 1169-78.

72. Tajuddin, N.F. and Druse, M.J., *In utero ethanol exposure decreased the density of serotonin neurons. Maternal ipsapirone treatment exerted a protective effect.* Brain Res Dev Brain Res, 1999. **117**(1): p. 91-7.

73. Zeisel, S.H., *Choline: needed for normal development of memory.* J Am Coll Nutr, 2000. **19**(5 Suppl): p. 528S-531S.

74. Thomas, J.D., et al., *Prenatal choline supplementation mitigates the adverse effects of prenatal alcohol exposure on development in rats.* Neurotoxicol Teratol, 2009. **31**(5): p. 303-11.

75. Thomas, J.D., et al., *Prenatal choline supplementation mitigates behavioral alterations associated with prenatal alcohol exposure in rats.* Birth Defects Res A Clin Mol Teratol, 2010. **88**(10): p. 827-37.

76. Boehme, F., et al., *Voluntary exercise induces adult hippocampal neurogenesis and BDNF expression in a rodent model of fetal alcohol spectrum disorders.* Eur J Neurosci, 2011. **33**(10): p. 1799-811.

77. Brocardo, P.S., et al., *Anxiety- and depression-like behaviors are accompanied by an increase in oxidative stress in a rat model of fetal alcohol spectrum disorders: Protective effects of voluntary physical exercise.* Neuropharmacology, 2012. **62**(4): p. 1607-18.

78. Thomas, J.D., et al., *Voluntary exercise influences behavioral development in rats exposed to alcohol during the neonatal brain growth spurt.* Behav Neurosci, 2008. **122**(6): p. 1264-73.

79. Famy, C., et al., *Mental illness in adults with fetal alcohol syndrome or fetal alcohol effects.* Am J Psychiatry, 1998. **155**(4): p. 552-4.

80. Barr, H.M., et al., *Binge drinking during pregnancy as a predictor of psychiatric disorders on the Structured Clinical Interview for DSM-IV in young adult offspring.* Am J Psychiatry, 2006. **163**(6): p. 1061-5.

81. Baldwin, M.R., *Fetal alcohol spectrum disorders and suicidality in a healthcare setting.* Int J Circumpolar Health, 2007. **66 Suppl 1**: p. 54-60.

82. Boyce, W.T., et al., *Psychobiologic reactivity to stress and childhood respiratory illnesses: results of two prospective studies.* Psychosom Med, 1995. **57**(5): p. 411-22.

83. Cohen, S. and Hamrick, N., *Stable individual differences in physiological response to stressors: implications for stress-elicited changes in immune related health.* Brain Behav Immun, 2003. **17**(6): p. 407-14.

84. Myin-Germeys, I., et al., *Emotional reactivity to daily life stress in psychosis and affective disorder: an experience sampling study.* Acta Psychiatr Scand, 2003. **107**(2): p. 124-31.

85. Myin-Germeys, I. and van Os, J., *Stress-reactivity in psychosis: evidence for an affective pathway to psychosis.* Clin Psychol Rev, 2007. **27**(4): p. 409-24.

86. Schlotz, W. and Phillips, D.I., *Fetal origins of mental health: evidence and mechanisms.* Brain Behav Immun, 2009. **23**(7): p. 905-16.

87. Matthews, S.G., *Early programming of the hypothalamo-pituitary-adrenal axis.* Trends Endocrinol Metab, 2002. **13**(9): p. 373-80.

88. Hellemans, K.G., et al., *Prenatal alcohol exposure increases vulnerability to stress and anxiety-like disorders in adulthood.* Ann N Y Acad Sci, 2008. **1144**: p. 154-75.

89. Weinberg, J., et al., *Prenatal alcohol exposure: foetal programming, the hypothalamic-pituitary-adrenal axis and sex differences in outcome.* J Neuroendocrinol, 2008. **20**(4): p. 470-88.

90. Lee, S., et al., *Increased activity of the hypothalamic-pituitary-adrenal axis of rats exposed to alcohol in utero: role of altered pituitary and hypothalamic function.* Mol Cell Neurosci, 2000. **16**(4): p. 515-28.

91. Weinberg, J., et al., *Fetal ethanol exposure: hypothalamic-pituitary-adrenal and beta-endorphin responses to repeated stress.* Alcohol Clin Exp Res, 1996. **20**(1): p. 122-31.

92. Taylor, A.N., et al., *Fetal exposure to ethanol enhances pituitary-adrenal and temperature responses to ethanol in adult rats.* Alcohol Clin Exp Res, 1981. **5**(2): p. 237-46.

93. Taylor, A.N., et al., *Maternal alcohol consumption and stress responsiveness in offspring.* Adv Exp Med Biol, 1988. **245**: p. 311-7.

94. Weinberg, J., *Prenatal ethanol exposure alters adrenocortical development of offspring.* Alcohol Clin Exp Res, 1989. **13**(1): p. 73-83.

95. Giberson, P.K. and Weinberg, J., *Effects of prenatal ethanol exposure and stress in adulthood on lymphocyte populations in rats.* Alcohol Clin Exp Res, 1995. **19**(5): p. 1286-94.

96. Angelogianni, P. and Gianoulakis, C., *Prenatal exposure to ethanol alters the ontogeny of the beta-endorphin response to stress.* Alcohol Clin Exp Res, 1989. **13**(4): p. 564-71.

97. Gottesfeld, Z., et al., *Prenatal ethanol exposure alters immune capacity and noradrenergic synaptic transmission in lymphoid organs of the adult mouse.* Neuroscience, 1990. **35**(1): p. 185-94.

98. Anderson, R.A., Jr., *Endocrine balance as a factor in the etiology of the fetal alcohol syndrome.* Neurobehav Toxicol Teratol, 1981. **3**(2): p. 89-104.

99. Weinberg, J. and Bezio, S., *Alcohol-induced changes in pituitary-adrenal activity during pregnancy.* Alcohol Clin Exp Res, 1987. **11**(3): p. 274-80.

100. Weinberg, J. and Gallo, P.V., *Prenatal ethanol exposure: pituitary-adrenal activity in pregnant dams and offspring.* Neurobehav Toxicol Teratol, 1982. **4**(5): p. 515-20.

101. Arishima, K., et al., *Maternal-foetal interrelations of plasma corticosterone concentrations at the end of gestation in the rat.* J Endocrinol, 1977. **72**(2): p. 239-40.

102. Levine, S. and Mullins, R.F., Jr., *Hormonal influences on brain organization in infant rats.* Science, 1966. **152**(3729): p. 1585-92.

103. Slone, J.L. and Redei, E.E., *Maternal alcohol and adrenalectomy: asynchrony of stress response and forced swim behavior.* Neurotoxicol Teratol, 2002. **24**(2): p. 173-8.

104.   Lee, S. and Rivier, C., *Administration of corticosterone to pregnant adrenalectomized dams does not alter the hypothalamic-pituitary-adrenal axis' activity of the offspring.* Mol Cell Neurosci, 1992. **3**(2): p. 118-23.

105.   Redei, E., et al., *Maternal adrenalectomy alters the immune and endocrine functions of fetal alcohol-exposed male offspring.* Endocrinology, 1993. **133**(2): p. 452-60.

106.   Lee, S., et al., *Effect of prenatal exposure to ethanol on the activity of the hypothalamic-pituitary-adrenal axis of the offspring: Importance of the time of exposure to ethanol and possible modulating mechanisms.* Mol Cell Neurosci, 1990. **1**(2): p. 168-77.

107.   Glavas, M.M., et al., *Effects of prenatal ethanol exposure on basal limbic-hypothalamic-pituitary-adrenal regulation: role of corticosterone.* Alcohol Clin Exp Res, 2007. **31**(9): p. 1598-610.

108.   Glavas, M.M., et al., *Effects of prenatal ethanol exposure on hypothalamic-pituitary-adrenal regulation after adrenalectomy and corticosterone replacement.* Alcohol Clin Exp Res, 2001. **25**(6): p. 890-7.

109.   Osborn, J.A., et al., *Fetal ethanol exposure alters pituitary-adrenal sensitivity to dexamethasone suppression.* Psychoneuroendocrinology, 1996. **21**(2): p. 127-43.

110.   Sarkar, D.K., et al., *Alcohol exposure during the developmental period induces beta-endorphin neuronal death and causes alteration in the opioid control of stress axis function.* Endocrinology, 2007. **148**(6): p. 2828-34.

111.   Govorko, D., et al., *Male germline transmits fetal alcohol adverse effect on hypothalamic proopiomelanocortin gene across generations.* Biol Psychiatry, 2012. **72**(5): p. 378-88.

112.   Del Arbol, J.L., et al., *[Plasma concentrations of beta-endorphins in the children of alcoholic patients].* An Med Interna, 2007. **24**(6): p. 273-7.

113.   Boyadjieva, N.I., et al., *Beta-endorphin neuronal cell transplant reduces corticotropin releasing hormone hyperresponse to lipopolysaccharide and eliminates natural killer cell functional deficiencies in fetal alcohol exposed rats.* Alcohol Clin Exp Res, 2009. **33**(5): p. 931-7.

114.   Church, M.W. and Gerkin, K.P., *Hearing disorders in children with fetal alcohol syndrome: findings from case reports.* Pediatrics, 1988. **82**(2): p. 147-54.

115.   Arjona, A., et al., *Fetal ethanol exposure disrupts the daily rhythms of splenic granzyme B, IFN-gamma, and NK cell cytotoxicity in adulthood.* Alcohol Clin Exp Res, 2006. **30**(6): p. 1039-44.

116.   Steven, W.M., et al., *The effects of maternal ethanol consumption on lactational transfer of immunity to Trichinella spiralis in rats.* Alcohol Clin Exp Res, 1992. **16**(5): p. 884-90.

117.   Seelig, L.L., Jr., et al., *Effects of maternal ethanol consumption on the subsequent development of immunity to Trichinella spiralis in rat neonates.* Alcohol Clin Exp Res, 1996. **20**(3): p. 514-22.

118.   Ewald, S.J. and Walden, S.M., *Flow cytometric and histological analysis of mouse thymus in fetal alcohol syndrome.* J Leukoc Biol, 1988. **44**(5): p. 434-40.

119.   Ewald, S.J. and Frost, W.W., *Effect of prenatal exposure to ethanol on development of the thymus.* Thymus, 1987. **9**(4): p. 211-5.

120.   Redei, E., et al., *Alcohol exposure in utero results in diminished T-cell function and alterations in brain corticotropin-releasing factor and ACTH content.* Alcohol Clin Exp Res, 1989. **13**(3): p. 439-43.

121.   Taylor, A.N., et al., *Thymocyte development in male fetal alcohol-exposed rats.* Alcohol Clin Exp Res, 1999. **23**(3): p. 465-70.

122.   Itoh, T. and Kasahara, S., *Development of Thy 1 positive rat thymocytes: analysis of fetal and neonatal thymocytes by flow microfluorometry.* Thymus, 1987. **9**(1): p. 13-23.

123. Adkins, B., *Developmental regulation of the intrathymic T cell precursor population.* J Immunol, 1991. **146**(5): p. 1387-93.

124. Hsiao, L., et al., *Differentiation and maturation of macrophages into interdigitating cells and their multicellular complex formation in the fetal and postnatal rat thymus.* Thymus, 1991. **17**(4): p. 219-35.

125. Bray, L.A., et al., *Effect of ethanol on development of fetal mouse thymocytes in organ culture.* Cell Immunol, 1993. **151**(1): p. 12-23.

126. Giberson, P.K. and Blakley, B.R., *Effect of postnatal ethanol exposure on expression of differentiation antigens of murine splenic lymphocytes.* Alcohol Clin Exp Res, 1994. **18**(1): p. 21-8.

127. Ewald, S.J. and Huang, C., *Lymphocyte populations and immune responses in mice prenatally exposed to ethanol.* Prog Clin Biol Res, 1990. **325**: p. 191-200.

128. Norman, D.C., et al., *Diminished proliferative response of con A-blast cells to interleukin 2 in adult rats exposed to ethanol in utero.* Alcohol Clin Exp Res, 1989. **13**(1): p. 69-72.

129. Weinberg, J. and Jerrells, T.R., *Suppression of immune responsiveness: sex differences in prenatal ethanol effects.* Alcohol Clin Exp Res, 1991. **15**(3): p. 525-31.

130. Jerrells, T.R. and Weinberg, J., *Influence of ethanol consumption on immune competence of adult animals exposed to ethanol in utero.* Alcohol Clin Exp Res, 1998. **22**(2): p. 391-400.

131. Grossmann, A., et al., *Immune function in offspring of nonhuman primates (Macaca nemestrina) exposed weekly to 1.8 g/kg ethanol during pregnancy: preliminary observations.* Alcohol Clin Exp Res, 1993. **17**(4): p. 822-7.

132. Giberson, P.K., et al., *The effect of cold stress on lymphocyte proliferation in fetal ethanol-exposed rats.* Alcohol Clin Exp Res, 1997. **21**(8): p. 1440-7.

133. Revskoy, S., et al., *Corticotropin-releasing hormone and proopiomelanocortin gene expression is altered selectively in the male rat fetal thymus by maternal alcohol consumption.* Endocrinology, 1997. **138**(1): p. 389-96.

134. Cohen, M.M., Jr., *Neoplasia and the fetal alcohol and hydantoin syndromes.* Neurobehav Toxicol Teratol, 1981. **3**(2): p. 161-2.

135. Azouz, E.M., et al., *Fetal alcohol syndrome and bilateral tibial exostoses. A case report.* Pediatr Radiol, 1993. **23**(8): p. 615-6.

136. Becker, H., et al., *[Fetal alcohol syndrome and malignant tumors].* Wien Klin Wochenschr, 1982. **94**(14): p. 364-5.

137. Zhang, L., et al., *Low levels of expression of cytochromes P-450 in normal and cancerous fetal pancreatic tissues of hamsters treated with NNK and/or ethanol.* Toxicol Sci, 2000. **56**(2): p. 313-23.

138. Gottesfeld, Z., et al., *Fetal alcohol exposure and adult tumorigenesis.* Alcohol, 1992. **9**(6): p. 465-71.

139. Taylor, A.N., et al., *Actions of alcohol on immunity and neoplasia in fetal alcohol exposed and adult rats.* Alcohol Alcohol Suppl, 1993. **2**: p. 69-74.

140. Murugan, S., et al., *Alcohol Exposure in Utero Increases Susceptibility to Prostate Tumorigenesis in Rat Offspring.* Alcohol Clin Exp Res, 2013.

141. Reichman, M.E., et al., *Effects of alcohol consumption on plasma and urinary hormone concentrations in premenopausal women.* J Natl Cancer Inst, 1993. **85**(9): p. 722-7.

142. Trichopoulos, D., *Hypothesis: does breast cancer originate in utero?* Lancet, 1990. **335**(8695): p. 939-40.

143. Potischman, N. and Troisi, R., *In-utero and early life exposures in relation to risk of breast cancer.* Cancer Causes Control, 1999. **10**(6): p. 561-73.

144. Hilakivi-Clarke, L., et al., *A maternal diet high in n - 6 polyunsaturated fats alters mammary gland development, puberty onset, and breast cancer risk among female rat offspring.* Proc Natl Acad Sci U S A, 1997. **94**(17): p. 9372-7.

145. Albrecht, W., *Words of wisdom. Re: maternal smoking, alcohol, and coffee use during pregnancy and son's risk of testicular cancer.* Eur Urol, 2010. **57**(2): p. 357-8.

146. Dokur, M., et al., *Beta-endorphin modulation of interferon-gamma, perforin and granzyme B levels in splenic NK cells: effects of ethanol.* J Neuroimmunol, 2005. **166**(1-2): p. 29-38.

147. Boyadjieva, N., et al., *Chronic ethanol inhibits NK cell cytolytic activity: role of opioid peptide beta-endorphin.* J Immunol, 2001. **167**(10): p. 5645-52.

148. Eipper, B.A. and Mains, R.E., *Structure and biosynthesis of pro-adrenocorticotropin/endorphin and related peptides.* Endocr Rev, 1980. **1**(1): p. 1-27.

149. Dores, R.M., et al., *Analyzing the evolution of the opioid/orphanin gene family.* Mass Spectrom Rev, 2002. **21**(4): p. 220-43.

150. Dores, R.M. and Baron, A.J., *Evolution of POMC: origin, phylogeny, posttranslational processing, and the melanocortins.* Ann N Y Acad Sci, 2011. **1220**: p. 34-48.

151. Santangelo, A.M., et al., *Ancient exaptation of a CORE-SINE retroposon into a highly conserved mammalian neuronal enhancer of the proopiomelanocortin gene.* PLoS Genet, 2007. **3**(10): p. 1813-26.

152. Bumaschny, V.F., et al., *Transcriptional regulation of pituitary POMC is conserved at the vertebrate extremes despite great promoter sequence divergence.* Mol Endocrinol, 2007. **21**(11): p. 2738-49.

153. Autelitano, D.J., et al., *Hormonal regulation of POMC gene expression.* Annu Rev Physiol, 1989. **51**: p. 715-26.

154. Takahashi, H., et al., *Isolation and structural organization of the human corticotropin--beta-lipotropin precursor gene.* FEBS Lett, 1981. **135**(1): p. 97-102.

155. Takahashi, H., et al., *Complete nucleotide sequence of the human corticotropin-beta-lipotropin precursor gene.* Nucleic Acids Res, 1983. **11**(19): p. 6847-58.

156. Newell-Price, J., *Proopiomelanocortin gene expression and DNA methylation: implications for Cushing's syndrome and beyond.* J Endocrinol, 2003. **177**(3): p. 365-72.

157. Civelli, O., et al., *Detection and quantitation of pro-opiomelanocortin mRNA in pituitary and brain tissues from different species.* J Biol Chem, 1982. **257**(12): p. 6783-7.

158. Drouin, J., et al., *Structure of the rat pro-opiomelanocortin (POMC) gene.* FEBS Lett, 1985. **193**(1): p. 54-8.

159. Millington, G.W., *The role of proopiomelanocortin (POMC) neurones in feeding behaviour.* Nutr Metab (Lond), 2007. **4**: p. 18.

160. Gee, C.E., et al., *Identification of proopiomelanocortin neurones in rat hypothalamus by in situ cDNA-mRNA hybridization.* Nature, 1983. **306**(5941): p. 374-6.

161. Chen, C.L., et al., *Expression of pro-opiomelanocortin-like gene in the testis and Leydig cell lines.* Ann N Y Acad Sci, 1984. **438**: p. 659-62.

162. Chen, C.L., et al., *Expression and regulation of proopiomelanocortin-like gene in the ovary and placenta: comparison with the testis.* Endocrinology, 1986. **118**(6): p. 2382-9.

163. Buzzetti, R., et al., *Expression of pro-opiomelanocortin gene and quantification of adrenocorticotropic hormone-like immunoreactivity in human normal peripheral mononuclear cells and lymphoid and myeloid malignancies.* J Clin Invest, 1989. **83**(2): p. 733-7.

164. Revskoy, S. and Gupta, D., *Ontogenetic studies of POMC gene expression in the immune organs in rats.* Neuroendocrinol Lett, 1997. **14**: p. 67-74.

165. DeBold, C.R., et al., *Ectopic pro-opiolipomelanocortin: sequence of cDNA coding for beta-melanocyte-stimulating hormone and beta-endorphin.* Science, 1983. **220**(4598): p. 721-3.

166. Smith, A.I. and Funder, J.W., *Proopiomelanocortin processing in the pituitary, central nervous system, and peripheral tissues.* Endocr Rev, 1988. **9**(1): p. 159-79.

167. Gagner, J.P. and Drouin, J., *Opposite regulation of pro-opiomelanocortin gene transcription by glucocorticoids and CRH.* Mol Cell Endocrinol, 1985. **40**(1): p. 25-32.

168. Plotsky, P.M., *Opioid inhibition of immunoreactive corticotropin-releasing factor secretion into the hypophysial-portal circulation of rats.* Regul Pept, 1986. **16**(3-4): p. 235-42.

169. Birnberg, N.C., et al., *Glucocorticoids regulate proopiomelanocortin gene expression in vivo at the levels of transcription and secretion.* Proc Natl Acad Sci U S A, 1983. **80**(22): p. 6982-6.

170. Wardlaw, S.L. and Frantz, A.G., *Brain beta-endorphin during pregnancy, parturition, and the postpartum period.* Endocrinology, 1983. **113**(5): p. 1664-8.

171. Zagon, I.S. and McLaughlin, P.J., *Behavioral effects of prenatal exposure to opiates.* Monogr Neural Sci, 1983. **9**: p. 159-68.

172. Sandman, C.A. and Yessaian, N., *Persisting subsensitivity of the striatal dopamine system after fetal exposure to beta-endorphin.* Life Sci, 1986. **39**(19): p. 1755-63.

173. Chen, L., et al., *Insulin resistance of gluconeogenic pathways in neonatal rats after prenatal ethanol exposure.* Am J Physiol Regul Integr Comp Physiol, 2004. **286**(3): p. R554-9.

174. Chen, L. and Nyomba, B.L., *Whole body insulin resistance in rat offspring of mothers consuming alcohol during pregnancy or lactation: comparing prenatal and postnatal exposure.* J Appl Physiol, 2004. **96**(1): p. 167-72.

175. O'Donohue, T.L. and Dorsa, D.M., *The opiomelanotropinergic neuronal and endocrine systems.* Peptides, 1982. **3**(3): p. 353-95.

176. Gianoulakis, C., *Effect of prenatal exposure to ethanol on body growth and the pituitary beta-endorphin.* Alcohol Clin Exp Res, 1987. **11**(6): p. 567-73.

177. Lee, S. and Rivier, C., *Prenatal alcohol exposure blunts interleukin-1-induced ACTH and beta-endorphin secretion by immature rats.* Alcohol Clin Exp Res, 1993. **17**(5): p. 940-5.

178. Bateman, A., et al., *The immune-hypothalamic-pituitary-adrenal axis.* Endocr Rev, 1989. **10**(1): p. 92-112.

179. Gianoulakis, C. and Barcomb, A., *Effect of acute ethanol in vivo and in vitro on the beta-endorphin system in the rat.* Life Sci, 1987. **40**(1): p. 19-28.

180. Sarkar, D.K. and Minami, S., *Effect of acute ethanol on beta-endorphin secretion from rat fetal hypothalamic neurons in primary cultures.* Life Sci, 1990. **47**(9): p. PL31-6.

181. Gianoulakis, C., *Characterization of the effects of acute ethanol administration on the release of beta-endorphin peptides by the rat hypothalamus.* Eur J Pharmacol, 1990. **180**(1): p. 21-9.

182. Boyadjieva, N.I. and Sarkar, D.K., *The role of cAMP in ethanol-regulated beta-endorphin release from hypothalamic neurons.* Alcohol Clin Exp Res, 1997. **21**(4): p. 728-31.

183. Boyadjieva, N.I. and Sarkar, D.K., *Role of microglia in ethanol's apoptotic action on hypothalamic neuronal cells in primary cultures.* Alcohol Clin Exp Res, 2010. **34**(11): p. 1835-42.

184. Bekdash, R.A., et al., *Gestational Choline Supplementation Normalized Fetal Alcohol-Induced Alterations in Histone Modifications, DNA Methylation, and*

Proopiomelanocortin (POMC) Gene Expression in beta-Endorphin-Producing POMC Neurons of the Hypothalamus. Alcohol Clin Exp Res, 2013.

185. Selye, H., *The evolution of the stress concept.* Am Sci, 1973. **61**(6): p. 692-9.
186. Yates, F.E., et al., *Application of control systems theory to physiology. Adrenal glucocorticoid control system.* Fed Proc, 1969. **28**(1): p. 71-83.
187. Cannon, W.B., *The Adrenal Medulla.* Bull N Y Acad Med, 1940. **16**(1): p. 3-13.
188. Chrousos, G.P. and Gold, P.W., *The concepts of stress and stress system disorders. Overview of physical and behavioral homeostasis.* JAMA, 1992. **267**(9): p. 1244-52.
189. Dhabhar, F.S. and McEwen, B.S., *Acute stress enhances while chronic stress suppresses cell-mediated immunity in vivo: a potential role for leukocyte trafficking.* Brain Behav Immun, 1997. **11**(4): p. 286-306.
190. Joels, M. and Baram, T.Z., *The neuro-symphony of stress.* Nat Rev Neurosci, 2009. **10**(6): p. 459-66.
191. Dayas, C.V., et al., *Stressor categorization: acute physical and psychological stressors elicit distinctive recruitment patterns in the amygdala and in medullary noradrenergic cell groups.* Eur J Neurosci, 2001. **14**(7): p. 1143-52.
192. Ulrich-Lai, Y.M. and Herman, J.P., *Neural regulation of endocrine and autonomic stress responses.* Nat Rev Neurosci, 2009. **10**(6): p. 397-409.
193. Fenoglio, K.A., et al., *Hippocampal neuroplasticity induced by early-life stress: functional and molecular aspects.* Front Neuroendocrinol, 2006. **27**(2): p. 180-92.
194. de Kloet, E.R., et al., *Stress and the brain: from adaptation to disease.* Nat Rev Neurosci, 2005. **6**(6): p. 463-75.
195. McEwen, B.S., *Physiology and neurobiology of stress and adaptation: central role of the brain.* Physiol Rev, 2007. **87**(3): p. 873-904.
196. McGaugh, J.L., *The amygdala modulates the consolidation of memories of emotionally arousing experiences.* Annu Rev Neurosci, 2004. **27**: p. 1-28.
197. Pecoraro, N., et al., *From Malthus to motive: how the HPA axis engineers the phenotype, yoking needs to wants.* Prog Neurobiol, 2006. **79**(5-6): p. 247-340.
198. Dayas, C.V., et al., *Medullary neurones regulate hypothalamic corticotropin-releasing factor cell responses to an emotional stressor.* Neuroscience, 2001. **105**(3): p. 707-19.
199. Chuluyan, H.E., et al., *Noradrenergic innervation of the hypothalamus participates in adrenocortical responses to interleukin-1.* Neuroendocrinology, 1992. **56**(1): p. 106-11.
200. Calogero, A.E., et al., *Pulsatile activation of the hypothalamic-pituitary-adrenal axis during major surgery.* Metabolism, 1992. **41**(8): p. 839-45.
201. Holmes, M.C., et al., *Magnocellular axons in passage through the median eminence release vasopressin.* Nature, 1986. **319**(6051): p. 326-9.
202. Buckingham, J.C., *The influence of corticosteroids on the secretion of corticotrophin and its hypothalamic releasing hormone.* J Physiol, 1979. **286**: p. 331-42.
203. Dallman, M.F., et al., *Characterization of corticosterone feedback regulation of ACTH secretion.* Ann N Y Acad Sci, 1987. **512**: p. 402-14.
204. Gillies, G.E., et al., *Corticotropin releasing activity of the new CRF is potentiated several times by vasopressin.* Nature, 1982. **299**(5881): p. 355-7.
205. Munck, A., et al., *Physiological functions of glucocorticoids in stress and their relation to pharmacological actions.* Endocr Rev, 1984. **5**(1): p. 25-44.
206. Bamberger, C.M., et al., *Molecular determinants of glucocorticoid receptor function and tissue sensitivity to glucocorticoids.* Endocr Rev, 1996. **17**(3): p. 245-61.
207. Charmandari, E., et al., *Endocrinology of the stress response.* Annu Rev Physiol, 2005. **67**: p. 259-84.

208. Ottenweller, J.E. and Meier, A.H., *Adrenal innervation may be an extrapituitary mechanism able to regulate adrenocortical rhythmicity in rats.* Endocrinology, 1982. **111**(4): p. 1334-8.

209. Calogero, A.E., et al., *Catecholamine effects upon rat hypothalamic corticotropin-releasing hormone secretion in vitro.* J Clin Invest, 1988. **82**(3): p. 839-46.

210. Valentino, R.J., et al., *Corticotropin-releasing factor activates noradrenergic neurons of the locus coeruleus.* Brain Res, 1983. **270**(2): p. 363-7.

211. Calogero, A.E., et al., *Multiple feedback regulatory loops upon rat hypothalamic corticotropin-releasing hormone secretion. Potential clinical implications.* J Clin Invest, 1988. **82**(3): p. 767-74.

212. Calogero, A.E., et al., *Mechanisms of serotonin receptor agonist-induced activation of the hypothalamic-pituitary-adrenal axis in the rat.* Endocrinology, 1990. **126**(4): p. 1888-94.

213. Fuller, R.W., *Serotonin receptors involved in regulation of pituitary-adrenocortical function in rats.* Behav Brain Res, 1996. **73**(1-2): p. 215-9.

214. Calogero, A.E., et al., *Interaction between GABAergic neurotransmission and rat hypothalamic corticotropin-releasing hormone secretion in vitro.* Brain Res, 1988. **463**(1): p. 28-36.

215. Overton, J.M. and Fisher, L.A., *Modulation of central nervous system actions of corticotropin-releasing factor by dynorphin-related peptides.* Brain Res, 1989. **488**(1-2): p. 233-40.

216. Keller-Wood, M.E. and Dallman, M.F., *Corticosteroid inhibition of ACTH secretion.* Endocr Rev, 1984. **5**(1): p. 1-24.

217. Roth, R.H., et al., *Stress and the mesocorticolimbic dopamine systems.* Ann N Y Acad Sci, 1988. **537**: p. 138-47.

218. Imperato, A., et al., *Changes in brain dopamine and acetylcholine release during and following stress are independent of the pituitary-adrenocortical axis.* Brain Res, 1991. **538**(1): p. 111-7.

219. Gray, T.S., et al., *Direct projections from the central amygdaloid nucleus to the hypothalamic paraventricular nucleus: possible role in stress-induced adrenocorticotropin release.* Neuroendocrinology, 1989. **50**(4): p. 433-46.

220. Goldfarb, A.H. and Jamurtas, A.Z., *Beta-endorphin response to exercise. An update.* Sports Med, 1997. **24**(1): p. 8-16.

221. Sarkar, D.K., et al., *Cyclic adenosine monophosphate differentiated beta-endorphin neurons promote immune function and prevent prostate cancer growth.* Proc Natl Acad Sci U S A, 2008. **105**(26): p. 9105-10.

222. Kawano, H. and Masuko, S., *Beta-endorphin-, adrenocorticotrophic hormone- and neuropeptide y-containing projection fibers from the arcuate hypothalamic nucleus make synaptic contacts on to nucleus preopticus medianus neurons projecting to the paraventricular hypothalamic nucleus in the rat.* Neuroscience, 2000. **98**(3): p. 555-65.

223. Boyadjieva, N., et al., *Role of beta-endorphin, corticotropin-releasing hormone, and autonomic nervous system in mediation of the effect of chronic ethanol on natural killer cell cytolytic activity.* Alcohol Clin Exp Res, 2006. **30**(10): p. 1761-7.

224. Eijgelshoven, M.H., et al., *Activation of glucocorticoid receptors and the effect of naloxone during hemorrhagic hypotension.* Eur J Pharmacol, 1991. **205**(2): p. 183-9.

225. Plotsky, P.M., et al., *Central and feedback regulation of hypothalamic corticotropin-releasing factor secretion.* Ciba Found Symp, 1993. **172**: p. 59-75; discussion 75-84.

226. Zangen, A., et al., *Impaired release of beta-endorphin in response to serotonin in a rat model of depression.* Neuroscience, 2002. **110**(3): p. 389-93.

227. Leonard, B.E. and Song, C., *Stress and the immune system in the etiology of anxiety and depression.* Pharmacol Biochem Behav, 1996. **54**(1): p. 299-303.

228. Zorrilla, E.P., et al., *The relationship of depression and stressors to immunological assays: a meta-analytic review.* Brain Behav Immun, 2001. **15**(3): p. 199-226.

229. Nunes, S.O., et al., *Immune and hormonal activity in adults suffering from depression.* Braz J Med Biol Res, 2002. **35**(5): p. 581-7.

230. Bloom, B.L., et al., *Marital disruption as a stressor: a review and analysis.* Psychol Bull, 1978. **85**(4): p. 867-94.

231. Kiecolt-Glaser, J.K., et al., *Marital quality, marital disruption, and immune function.* Psychosom Med, 1987. **49**(1): p. 13-34.

232. Monjan, A.A. and Collector, M.I., *Stress-induced modulation of the immune response.* Science, 1977. **196**(4287): p. 307-8.

233. Moynihan, J.A., *Mechanisms of stress-induced modulation of immunity.* Brain Behav Immun, 2003. **17 Suppl 1**: p. S11-6.

234. Ader, R., et al., *Psychoneuroimmunology: interactions between the nervous system and the immune system.* Lancet, 1995. **345**(8942): p. 99-103.

235. Blalock, J.E., *The syntax of immune-neuroendocrine communication.* Immunol Today, 1994. **15**(11): p. 504-11.

236. Reiche, E.M., et al., *Stress, depression, the immune system, and cancer.* Lancet Oncol, 2004. **5**(10): p. 617-25.

237. Altemus, M., et al., *Stress-induced changes in skin barrier function in healthy women.* J Invest Dermatol, 2001. **117**(2): p. 309-17.

238. Schwab, C.L., et al., *Modeling and predicting stress-induced immunosuppression in mice using blood parameters.* Toxicol Sci, 2005. **83**(1): p. 101-13.

239. Benschop, R.J., et al., *Catecholamine-induced leukocytosis: early observations, current research, and future directions.* Brain Behav Immun, 1996. **10**(2): p. 77-91.

240. Sanders, V.M. and Straub, R.H., *Norepinephrine, the beta-adrenergic receptor, and immunity.* Brain Behav Immun, 2002. **16**(4): p. 290-332.

241. Dokur, M., et al., *Catecholaminergic control of NK cell cytolytic activity regulatory factors in the spleen.* J Neuroimmunol, 2004. **151**(1-2): p. 148-57.

242. Black, P.H., *Central nervous system-immune system interactions: psychoneuroendocrinology of stress and its immune consequences.* Antimicrob Agents Chemother, 1994. **38**(1): p. 1-6.

243. Webster, J.I., et al., *Neuroendocrine regulation of immunity.* Annu Rev Immunol, 2002. **20**: p. 125-63.

244. Kin, N.W. and Sanders, V.M., *It takes nerve to tell T and B cells what to do.* J Leukoc Biol, 2006. **79**(6): p. 1093-104.

245. Sternberg, E.M., *Neural regulation of innate immunity: a coordinated nonspecific host response to pathogens.* Nat Rev Immunol, 2006. **6**(4): p. 318-28.

246. Herberman, R.B. and Ortaldo, J.R., *Natural killer cells: their roles in defenses against disease.* Science, 1981. **214**(4516): p. 24-30.

247. Graubert, T.A., et al., *The role of granzyme B in murine models of acute graft-versus-host disease and graft rejection.* Blood, 1996. **87**(4): p. 1232-7.

248. Madden, K.S., et al., *Catecholamine influences and sympathetic neural modulation of immune responsiveness.* Annu Rev Pharmacol Toxicol, 1995. **35**: p. 417-48.

249. Irwin, M., et al., *Sympathetic nervous system mediates central corticotropin-releasing factor induced suppression of natural killer cytotoxicity.* J Pharmacol Exp Ther, 1990. **255**(1): p. 101-7.

250. Dokur, M., et al., *Modulation of hypothalamic beta-endorphin-regulated expression of natural killer cell cytolytic activity regulatory factors by ethanol in male Fischer-344 rats.* Alcohol Clin Exp Res, 2004. **28**(8): p. 1180-6.

251. Boyadjieva, N.I., et al., *Beta-endorphin modulation of lymphocyte proliferation: effects of ethanol.* Alcohol Clin Exp Res, 2002. **26**(11): p. 1719-27.

252. Shahabi, N.A., et al., *Murine splenocytes express a naloxone-insensitive binding site for beta-endorphin.* Endocrinology, 1990. **126**(3): p. 1442-8.

253. Mathews, P.M., et al., *Enhancement of natural cytotoxicity by beta-endorphin.* J Immunol, 1983. **130**(4): p. 1658-62.

254. Gatti, G., et al., *Interplay in vitro between ACTH, beta-endorphin, and glucocorticoids in the modulation of spontaneous and lymphokine-inducible human natural killer (NK) cell activity.* Brain Behav Immun, 1993. **7**(1): p. 16-28.

255. Levav, I., et al., *Cancer incidence and survival following bereavement.* Am J Public Health, 2000. **90**(10): p. 1601-7.

256. Rustoen, T., et al., *The importance of hope as a mediator of psychological distress and life satisfaction in a community sample of cancer patients.* Cancer Nurs, 2010. **33**(4): p. 258-67.

257. Spiegel, D. and Sephton, S.E., *Psychoneuroimmune and endocrine pathways in cancer: effects of stress and support.* Semin Clin Neuropsychiatry, 2001. **6**(4): p. 252-65.

258. Thaker, P.H., et al., *Chronic stress promotes tumor growth and angiogenesis in a mouse model of ovarian carcinoma.* Nat Med, 2006. **12**(8): p. 939-44.

259. Visintainer, M.A., et al., *Tumor rejection in rats after inescapable or escapable shock.* Science, 1982. **216**(4544): p. 437-9.

260. Laudenslager, M.L., et al., *Coping and immunosuppression: inescapable but not escapable shock suppresses lymphocyte proliferation.* Science, 1983. **221**(4610): p. 568-70.

261. Glaser, R., et al., *Effects of stress on methyltransferase synthesis: an important DNA repair enzyme.* Health Psychol, 1985. **4**(5): p. 403-12.

262. Fischman, H.K. and Kelly, D.D., *Sister chromatid exchanges induced by behavioral stress.* Ann N Y Acad Sci, 1987. **496**: p. 426-35.

263. Kiecolt-Glaser, J.K., et al., *Distress and DNA repair in human lymphocytes.* J Behav Med, 1985. **8**(4): p. 311-20.

264. Kaprara, A., et al., *The corticotropin releasing factor system in cancer: expression and pathophysiological implications.* Cell Mol Life Sci, 2010. **67**(8): p. 1293-306.

265. Wang, J. and Li, S., *Corticotropin-releasing factor family and its receptors: tumor therapeutic targets?* Biochem Biophys Res Commun, 2007. **362**(4): p. 785-8.

266. Sastry, K.S., et al., *Epinephrine protects cancer cells from apoptosis via activation of cAMP-dependent protein kinase and BAD phosphorylation.* J Biol Chem, 2007. **282**(19): p. 14094-100.

267. Sloan, E.K., et al., *The sympathetic nervous system induces a metastatic switch in primary breast cancer.* Cancer Res, 2010. **70**(18): p. 7042-52.

268. Schleifer, S.J., et al., *Immunity in young adults with major depressive disorder.* Am J Psychiatry, 1996. **153**(4): p. 477-82.

269. Ben-Eliyahu, S., et al., *Evidence that stress and surgical interventions promote tumor development by suppressing natural killer cell activity.* Int J Cancer, 1999. **80**(6): p. 880-8.

270. Wu, W., et al., *Social isolation stress enhanced liver metastasis of murine colon 26-L5 carcinoma cells by suppressing immune responses in mice.* Life Sci, 2000. **66**(19): p. 1827-38.

271. Wu, W., et al., *Social isolation stress impairs the resistance of mice to experimental liver metastasis of murine colon 26-L5 carcinoma cells.* Biol Pharm Bull, 2001. **24**(7): p. 772-6.

272. Wu, W., et al., *Social isolation stress augments angiogenesis induced by colon 26-L5 carcinoma cells in mice.* Clin Exp Metastasis, 2000. **18**(1): p. 1-10.

273. Colson, Y.L. and Grinstaff, M.W., *Biologically responsive polymeric nanoparticles for drug delivery.* Adv Mater, 2012. **24**(28): p. 3878-86.

274. Begley, D.J., *The blood-brain barrier: principles for targeting peptides and drugs to the central nervous system.* J Pharm Pharmacol, 1996. **48**(2): p. 136-46.

275. Kreuter, J., *Nanoparticulate systems for brain delivery of drugs.* Adv Drug Deliv Rev, 2001. **47**(1): p. 65-81.

276. Costantino, L., *Drug delivery to the CNS and polymeric nanoparticulate carriers.* Future Med Chem, 2010. **2**(11): p. 1681-701.

277. Agarwal, A., et al., *Nanoparticles as novel carrier for brain delivery: a review.* Curr Pharm Des, 2009. **15**(8): p. 917-25.

278. Kipp, J.E., *The role of solid nanoparticle technology in the parenteral delivery of poorly water-soluble drugs.* Int J Pharm, 2004. **284**(1-2): p. 109-22.

279. Brannon-Peppas, L. and Blanchette, J.O., *Nanoparticle and targeted systems for cancer therapy.* Adv Drug Deliv Rev, 2004. **56**(11): p. 1649-59.

280. McKeon, R.J., et al., *Reduction of neurite outgrowth in a model of glial scarring following CNS injury is correlated with the expression of inhibitory molecules on reactive astrocytes.* J Neurosci, 1991. **11**(11): p. 3398-411.

281. Kim, W.Y. and Snider, W.D., *Neuroscience. Overcoming inhibitions.* Science, 2008. **322**(5903): p. 869-72.

282. Kim, G., et al., *Activation of protein kinase A induces neuronal differentiation of HiB5 hippocampal progenitor cells.* Brain Res Mol Brain Res, 2002. **109**(1-2): p. 134-45.

283. Qiu, J., et al., *A role for cAMP in regeneration during development and after injury.* Prog Brain Res, 2002. **137**: p. 381-7.

284. Qiu, J., et al., *Spinal axon regeneration induced by elevation of cyclic AMP.* Neuron, 2002. **34**(6): p. 895-903.

285. Monsul, N.T., et al., *Intraocular injection of dibutyryl cyclic AMP promotes axon regeneration in rat optic nerve.* Exp Neurol, 2004. **186**(2): p. 124-33.

286. Martino, G. and Pluchino, S., *The therapeutic potential of neural stem cells.* Nat Rev Neurosci, 2006. **7**(5): p. 395-406.

287. Bauer, H.C., et al., *Neuronal stem cells in adults.* Exp Gerontol, 2006. **41**(2): p. 111-6.

288. Kim, H., et al., *Effects of dibutyryl cyclic-AMP on survival and neuronal differentiation of neural stem/progenitor cells transplanted into spinal cord injured rats.* PLoS One, 2011. **6**(6): p. e21744.

289. Mena, M.A., et al., *Effects of dibutyryl cyclic AMP and retinoic acid on the differentiation of dopamine neurons: prevention of cell death by dibutyryl cyclic AMP.* J Neurochem, 1995. **65**(6): p. 2612-20.

290. Tojima, T., et al., *Dual role of cyclic AMP-dependent protein kinase in neuritogenesis and synaptogenesis during neuronal differentiation.* J Neurosci Res, 2003. **74**(6): p. 829-37.

291. Zahir, T., et al., *Neural stem/progenitor cells differentiate in vitro to neurons by the combined action of dibutyryl cAMP and interferon-gamma.* Stem Cells Dev, 2009. **18**(10): p. 1423-32.

292. Hatton, J.D. and Hoi, S.U., *In vitro differentiation inhibits the migration of cultured neonatal rat cortical astrocytes transplanted to the neonatal rat cerebrum.* Int J Dev Neurosci, 1993. **11**(5): p. 583-94.

293. van Eerdenburg, F.J. and Rakic, P., *Early neurogenesis in the anterior hypothalamus of the rhesus monkey.* Brain Res Dev Brain Res, 1994. **79**(2): p. 290-6.

294. Magnus, T. and Rao, M.S., *Neural stem cells in inflammatory CNS diseases: mechanisms and therapy.* J Cell Mol Med, 2005. **9**(2): p. 303-19.

295. Baumann, M.D., et al., *An injectable drug delivery platform for sustained combination therapy.* J Control Release, 2009. **138**(3): p. 205-13.

296. Parr, A.M., et al., *Transplantation of adult rat spinal cord stem/progenitor cells for spinal cord injury.* J Neurotrauma, 2007. **24**(5): p. 835-45.

297. Karimi-Abdolrezaee, S., et al., *Delayed transplantation of adult neural precursor cells promotes remyelination and functional neurological recovery after spinal cord injury.* J Neurosci, 2006. **26**(13): p. 3377-89.

298. Rooney, G.E., et al., *Sustained delivery of dibutyryl cyclic adenosine monophosphate to the transected spinal cord via oligo [(polyethylene glycol) fumarate] hydrogels.* Tissue Eng Part A, 2011. **17**(9-10): p. 1287-302.

299. Society, A.C., *Cancer Facts & Figures 2009*, in *American cancer society*. 2009. p. 9-10.

300. Montgomery, M. and McCrone, S.H., *Psychological distress associated with the diagnostic phase for suspected breast cancer: systematic review.* J Adv Nurs, 2010. **66**(11): p. 2372-90.

301. Thaker, P.H. and Sood, A.K., *Neuroendocrine influences on cancer biology.* Semin Cancer Biol, 2008. **18**(3): p. 164-70.

302. Marchetti, B., et al., *A potential role for catecholamines in the development and progression of carcinogen-induced mammary tumors: hormonal control of beta-adrenergic receptors and correlation with tumor growth.* J Steroid Biochem Mol Biol, 1991. **38**(3): p. 307-20.

303. Abo, T. and Kawamura, T., *Immunomodulation by the autonomic nervous system: therapeutic approach for cancer, collagen diseases, and inflammatory bowel diseases.* Ther Apher, 2002. **6**(5): p. 348-57.

304. Lissoni, P., et al., *Evidence for altered opioid activity in patients with cancer.* Br J Cancer, 1987. **56**(6): p. 834-7.

305. Grinshpoon, A., et al., *Cancer in schizophrenia: is the risk higher or lower?* Schizophr Res, 2005. **73**(2-3): p. 333-41.

306. Page, G.G., et al., *Male--female differences in the impact of beta-adrenoceptor stimulation on resistance to experimental metastasis: exploring the effects of age and gonadal hormone involvement.* J Neuroimmunol, 2008. **193**(1-2): p. 113-9.

307. Wu, C.H., et al., *Nicotinic acetylcholine receptor-based blockade: applications of molecular targets for cancer therapy.* Clin Cancer Res, 2011. **17**(11): p. 3533-41.

308. Pensa, S., et al., *Stat3 and the inflammation/acute phase response in involution and breast cancer.* J Mammary Gland Biol Neoplasia, 2009. **14**(2): p. 121-9.

309. Wu, Y., et al., *Stabilization of snail by NF-kappaB is required for inflammation-induced cell migration and invasion.* Cancer Cell, 2009. **15**(5): p. 416-28.

310. Yoshida, J., et al., *Changes in the expression of E-cadherin repressors, Snail, Slug, SIP1, and Twist, in the development and progression of ovarian carcinoma: the important role of Snail in ovarian tumorigenesis and progression.* Med Mol Morphol, 2009. **42**(2): p. 82-91.

311. de Herreros, A.G., et al., *Snail family regulation and epithelial mesenchymal transitions in breast cancer progression.* J Mammary Gland Biol Neoplasia, 2010. **15**(2): p. 135-47.
312. Ben-Eliyahu, S., et al., *Acute alcohol intoxication suppresses natural killer cell activity and promotes tumor metastasis.* Nat Med, 1996. **2**(4): p. 457-60.
313. Jaiswal, S., et al., *Macrophages as mediators of tumor immunosurveillance.* Trends Immunol, 2010. **31**(6): p. 212-9.
314. Yang, Q., et al., *Antitumor activity of NK cells.* Immunol Res, 2006. **36**(1-3): p. 13-25.
315. Hazlett, L.D. and Hendricks, R.L., *Reviews for immune privilege in the year 2010: immune privilege and infection.* Ocul Immunol Inflamm, 2010. **18**(4): p. 237-43.
316. Young, L.H., et al., *How lymphocytes kill.* Annu Rev Med, 1990. **41**: p. 45-54.
317. O'Connor, G.M., et al., *Putting the natural killer cell in its place.* Immunology, 2006. **117**(1): p. 1-10.
318. Cox, J.H. and deSouza, M., *Evaluation of natural killer cell activity.* Mol Biotechnol, 2000. **15**(2): p. 147-54.
319. Kyriakides, T.R., et al., *The CC chemokine ligand, CCL2/MCP1, participates in macrophage fusion and foreign body giant cell formation.* Am J Pathol, 2004. **165**(6): p. 2157-66.
320. Kaler, P., et al., *Macrophage-derived IL-1beta stimulates Wnt signaling and growth of colon cancer cells: a crosstalk interrupted by vitamin D3.* Oncogene, 2009. **28**(44): p. 3892-902.
321. Miyazaki, A., et al., *Granulocyte macrophage colony-stimulating factor plays a priming role in murine macrophage growth induced by oxidized low density lipoprotein.* Ann N Y Acad Sci, 2000. **902**: p. 342-6.
322. Gregory, J.L., et al., *Macrophage migration inhibitory factor induces macrophage recruitment via CC chemokine ligand 2.* J Immunol, 2006. **177**(11): p. 8072-9.
323. Li, W., et al., *Modulation of innate immunity by IL-18.* J Reprod Immunol, 2009. **83**(1-2): p. 101-5.
324. Patel, N.S., et al., *Inflammatory cytokine levels correlate with amyloid load in transgenic mouse models of Alzheimer's disease.* J Neuroinflammation, 2005. **2**(1): p. 9.
325. Imura, H., et al., *Biosynthesis and distribution of opioid peptides.* J Endocrinol Invest, 1983. **6**(2): p. 139-49.
326. Gorelik, E., et al., *Role of NK cells in the control of metastatic spread and growth of tumor cells in mice.* Int J Cancer, 1982. **30**(1): p. 107-12.
327. Talmadge, J.E., et al., *Role of natural killer cells in tumor growth and metastasis: C57BL/6 normal and beige mice.* J Natl Cancer Inst, 1980. **65**(5): p. 929-35.
328. Nüssler, N.C., et al., *Reduced NK-Cell Activity in Patients with Metastatic Colon Cancer.* EXCLI Journal, 2007. **6**: p. 1-9.
329. Fulton, A., et al., *Relationship of natural killer cytotoxicity to clinical and biochemical parameters of primary human breast cancer.* Breast Cancer Res Treat, 1984. **4**(2): p. 109-16.
330. Garner, W.L., et al., *Human breast cancer and impaired NK cell function.* J Surg Oncol, 1983. **24**(1): p. 64-6.
331. Wakimoto, H., et al., *Effects of innate immunity on herpes simplex virus and its ability to kill tumor cells.* Gene Ther, 2003. **10**(11): p. 983-90.
332. Von Ah, D., et al., *Stress, optimism, and social support: impact on immune responses in breast cancer.* Res Nurs Health, 2007. **30**(1): p. 72-83.
333. Mandler, R.N., et al., *beta-Endorphin augments the cytolytic activity and interferon production of natural killer cells.* J Immunol, 1986. **136**(3): p. 934-9.

334.   Dunn, G.P., et al., *The immunobiology of cancer immunosurveillance and immunoediting.* Immunity, 2004. **21**(2): p. 137-48.
335.   Bryant, J., et al., *Calculation of lytic units for the expression of cell-mediated cytotoxicity.* J Immunol Methods, 1992. **146**(1): p. 91-103.
336.   Sarkar, D.K., et al., *Transplantation of beta-endorphin neurons into the hypothalamus promotes immune function and restricts the growth and metastasis of mammary carcinoma.* Cancer Res, 2011. **71**(19): p. 6282-91.
337.   Mothe, A.J., et al., *Neural stem/progenitor cells from the adult human spinal cord are multipotent and self-renewing and differentiate after transplantation.* PLoS One, 2011. **6**(11): p. e27079.
338.   Settle, M., *The hypothalamus.* Neonatal Netw, 2000. **19**(6): p. 9-14.
339.   Dworkin, S. and Mantamadiotis, T., *Targeting CREB signalling in neurogenesis.* Expert Opin Ther Targets, 2010. **14**(8): p. 869-79.
340.   Barlozzari, T., et al., *Direct evidence for the role of LGL in the inhibition of experimental tumor metastases.* J Immunol, 1985. **134**(4): p. 2783-9.
341.   Shakhar, G. and Ben-Eliyahu, S., *In vivo beta-adrenergic stimulation suppresses natural killer activity and compromises resistance to tumor metastasis in rats.* J Immunol, 1998. **160**(7): p. 3251-8.
342.   Ben-Eliyahu, S., et al., *Stress, NK cells, and cancer: Still a promissory note.* Brain Behav Immun, 2007. **21**(7): p. 881-7.
343.   Schuurman, H.J., *The nude rat.* Hum Exp Toxicol, 1995. **14**(1): p. 122-5.
344.   Dhabhar, F.S., *Stress, leukocyte trafficking, and the augmentation of skin immune function.* Ann N Y Acad Sci, 2003. **992**: p. 205-17.
345.   Neeman, E., et al., *Stress and skin leukocyte trafficking as a dual-stage process.* Brain Behav Immun, 2012. **26**(2): p. 267-76.
346.   Gupta, M.A., *Review of somatic symptoms in post-traumatic stress disorder.* Int Rev Psychiatry, 2013. **25**(1): p. 86-99.
347.   Sorensen, G.V., et al., *Use of beta-blockers, angiotensin-converting enzyme inhibitors, angiotensin II receptor blockers, and risk of breast cancer recurrence: a Danish nationwide prospective cohort study.* J Clin Oncol, 2013. **31**(18): p. 2265-72.
348.   Hazeldine, V., *Pharmacological management of acute asthma exacerbations in adults.* Nurs Stand, 2013. **27**(33): p. 43-9; quiz 51.
349.   Tseng, L.F., et al., *Beta-Endorphin as a potent analgesic by intravenous injection.* Nature, 1976. **263**(5574): p. 239-40.
350.   Loh, H.H., et al., *beta-endorphin is a potent analgesic agent.* Proc Natl Acad Sci U S A, 1976. **73**(8): p. 2895-8.

CURRICULUM   VITAE

9   Curriculum Vitae

CHANGQING ZHANG

EDUCATION
2007−2013   Ph.D. Candidate
Endocrinology & Animal Biosciences Program,
Rutgers, The State University of New Jersey, New Brunswick, NJ

2003−2007   B.S. in Biological Science
College of Life Science, *Peking University*, Beijing, China

RESEARCH EXPERIENCE
2007−2013   **Endocrine Research Facility, Department of Animal Sciences**
Rutgers University. Advisor: Dr. Dipak K. Sarkar

2005−2007   **National Laboratory of Biomembrane & Membrane Biotechnology**
College of Life Sciences, Peking University. Adviser: Shiqiang Wang

TEACHING EXPERIENCE
Spring 2009 **Department of Animal Sciences, Rutgers University**
Teaching Assistant: Animal Microtechniques and Tissue Culture Lab

Fall 2008   **Department of Animal Sciences, Rutgers University**
Teaching Assistant: Animal Nutrition

PUBLICATIONS

1. **Zhang C,** Murugan S, Boyadjieva N, Sarkar DK. Cancer chemoprevention by beta-endorphin neurons. (in communication with *Nature Medicine*).

2. **Zhang C**, Sarkar DK. β-endorphin neuron transplantation: a possible novel therapy for cancer prevention. *OncoImmunology 2012 July 1(4): 552-4.*

3. Maria A, Gangisetty O, **Zhang C**, Shrivastava P, Logan R, Sarkar DK. Fetal alcohol exposure disrupts metabolic signaling in hypothalamic proopiomelanocortin neurons via a circadian mechanism. (in communication with *Journal of Neuroscience*)

4. Murugan S, **Zhang C**, Mojtehedzadeh S, Sarkar DK. Alcohol exposure in utero induces histophysiological changes in prostate and increases susceptibility to prostate tumorigenesis inrat offspring. *Alcoholism 2013 Jul 26. doi: 10.1111/acer.12171. [Epub ahead of print]*

5. Sarkar DK, **Zhang C**. Beta-endorphin neuron regulates stress response and innate immunity to prevent breast cancer growth and progression. Vitamins and Hormones 2013 93: 263-76.

6. Bekdash RA, **Zhang C**, Sarkar DK. Gestational choline supplementation normalized fetal alcohol-induced alterations in histone modifications, DNA methylation, and proopiomelanocortin (POMC) gene expression in β-endorphin-producing POMC neurons of the hypothalamus. *Alcoholism 2013 July 37(7).*

7. Govorko D, Bekdash R, **Zhang C**, Sarkar DK. Male germline transmits fetal alcohol effect on the hypothalamic proopiomelanocortin gene across generations. *Biological Psychiatry 2012 Sep 1;72(5):378-88.* **(First three authors have equal contribution)**

8. Sarkar DK, Sengupta A, **Zhang C**, Boyadjieva N, Murugan S. Opiate antagonist prevents mu and delta opiate receptor dimerization to facilitate an agonist's ability to control ethanol-altered natural killer cell functions and mammary tumor growth. *Journal of Biological Chemistry 2012 May 11;287(20):16734-47.*

9. Logan RW, **Zhang C**, Murugan S, O'Connell S, Levitt D, Rosenwasser AM, Sarkar DK. Chronic shift-lag alters the circadian clock of natural killer cells and promotes lung cancer growth in rats. *Journal of Immunology 2012 Mar 15; 188(6):2583-91.*

10. Sarkar DK, Murugan S, **Zhang C**, Boyadjieva N. Regulation of Cancer Progression by β-Endorphin Neuron. *Cancer Research 2012 Feb 15; 72(4):836-40.*

11. Sarkar DK, **Zhang C**, Murugan S, Dokur M, Boyadjieva NI, Ortiguela M, Mojtehedzadeh S. Transplantation of β-endorphin neurons into the hypothalamus promotes immune function and restricts the growth and metastasis of mammary carcinoma. *Cancer Research 2011 71(19):6282-91.*

Meeting Abstracts:

1. **Zhang C**, Boyadjieva N, Ortiguela M, Franklin T, Sarkar DK. Fetal alcohol exposure increases tumor colonization in the lung: role of hypothalamic beta-endorphin. 35th Annual RSA Scientific Meeting, San Francisco, California, June 23-27, 2012.
2. Logan RW, **Zhang C**, O'Connel S, Ortiguela M, Boyadjieva N, Wynne O, Sarkar DK. Beta-endorphin neuron transplantation in the hypothalamus attenuates corticosterone response and anxiety-like behaviors in fetal alcohol exposed rats. (In press) 35th Annual RSA Scientific Meeting, San Francisco, California, June 23-27, 2012.
2. **Zhang C**, Murugan S, Boyadjieva N, Ortiguela M, Mojtehedzadeh S and Sarkar D. Transplantation of β-endorphin neurons into the hypothalamus inhibits prostate cancer growth in fetal alcohol exposed rats. 2012 Annual Retreat on Cancer Research in New Jersey, May 24, 2012.

3. Murugan S, **Zhang C**, Mojtehedzadeh S, Reuhl K, Sarkar DK.Alcohol exposure in utero induces histophysiological changes in prostate and increases susceptibility to prostate tumorigenesis in rat offspring. American Association of Cancer Research 103rd Annual Meeting, Chicago, IL, Mar 31, 2012.

4. Sarkar DK, **Zhang C**, Bekdash RA, Govorko D (2011) Postnatal treatment with azacytidine or trichostatin prevents prenatal ethanol-induced alteration in stress axis functions in the adult. *Alcoholism: Clinical and Experimental Research (supplement)* 35:6.

5. Govorko D, **Zhang C**, Sarkar DK (2011) Proopiomelanocortin gene hypermethylation is associated with its increased binding with methyl CpG binding protein 2 in the hypothalamus of fetal alcohol exposed rats. *Alcoholism: Clinical and Experimental Research (supplement)* 35:6.

6. Sarkar DK, **Zhang C**, Dokur M, Boyadjieva NI, Ortiguela M, Rafferty N, Mojtehedzadeh S, Reuhl K, Murugan S (2011) Beta-endorphin neuron transplants suppress mammary tumor gowth and progression via neuroimmune activation. American Association of Cancer Research 102nd Annual Meeting, Orlando, FL, Apr 4, 2011.

7. **Zhang C**, Mojtehedzadeh S, Ortiguela M, Sarkar DK (2010) Implantation of cyclic adenosine monophosphate delivering nanospheres into the third ventricle reduces metastasis of cancer cells into the lung in Fischer-344 rats. Society for Neuroscience 40th Annual Meeting, San Diego, CA, Nov 17, 2010.

8. Sengupta A, **Zhang C**, Boyadjieva N, Murugan S, Sarkar DK (2010) Naltrexone inhibits heterodimerization between Mu and Delta opiate receptors on splenocytes to increase delta agonist response and inhibit alcohol suppression of NK cell functions. Society for Neuroscience 40th Annual Meeting, San Diego, CA, Nov 17, 2010.

9. **Zhang C**, Govorko D, Bekdash RA, Sarkar DK (2010) Sex difference in the fetal alcohol-induced transgenerational changes in the basal and lipopolysaccaride-activated plasma corticosterone levels. *Alcoholism: Clinical and Experimental Research (supplement)* 34:113.

10. Govorko D, Bekdash RA, **Zhang C**, Sarkar DK (2010) Transgenerational epigenetic effects of fetal alcohol on promoter DNA methylation and gene expression of hypothalamic proopiomelanocortin. *Alcoholism: Clinical and Experimental Research (supplement)* 34:112.

11. Mojtahedzadeh S, **Zhang C**, Clement EM, Sarkar DK (2010) Alcohol exposure *in utero* leads to enhanced susceptibility to prostate tumorigenesis in rat offspring. *Alcoholism: Clinical and Experimental Research (supplement)* 34:141.

12. **Zhang C**, Boyadjieva NI, Ortiguela M, Sarkar DK (2009) Prenatal alcohol exposures reduces progenitor cells differentiation to BEP neurons while nanosphere's delivered cAMP increases BEP neuron growth. *Alcoholism: Clinical and Experimental Research (supplement)* 34:497.

13. Boyadjieva NI, **Zhang C**, Ortiguela M, Sarkar DK. (2009) Neural stem cell-derived beta-endorphin neuron transplants into the brain increase natural killer cell activity, decrease antiinflammatory cytokines and prevent metastatic colonization in rats. National Meeting for the American Association of Cancer research, Denver, CO, April 2009.