THE ROLE OF PERIOD 2 GENE IN MEDIATING ALCOHOL EFFECTS ON METABOLIC SIGNALS IN PROOPIOMELANOCORTIN PRODUCING NEURONS

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ABSTRACT OF THE DISSERTATION

The role of Period 2 gene in mediating alcohol effects on metabolic signals in proopiomelanocortin producing neurons

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An increasing body of knowledge suggests that fetal alcohol exposure disrupts circadian homeostasis and metabolic functions. In order to determine the mechanism by which fetal alcohol exposure alters the metabolic functions, we determined the postnatal ethanol-induced changes in the function of Proopiomelanocortin (*Pomc*) neurons known to regulate metabolic homeostasis. Additionally, we evaluated the role of a clock regulatory gene, *Period 2* (*Per*2), in mediation of alcohol effects on *Pomc* neurons. To determine the interaction between alcohol and *Per*2 on β-endorphin neuronal activity, we first measured the secretion of this peptide from mediobasal hypothalamic (MBH) cells of postnatal mice in primary cultures following treatment with ethanol for various time periods. We found that ethanol acutely stimulated but chronically inhibited β-endorphin secretion *in vitro* and these effects of ethanol on β-endorphin secretion were absent following *Per*2 mutation. We also evaluated the effect of the clock gene mutation on postnatal alcohol programming of *Pomc* and metabolic genes expression in the hypothalamus. For this, We measured the levels of *Pomc* and various metabolic genes (*Stat*3, *Sirt*1, *Asb4* and *Pgc*1α) and *Pomc* gene products β-endorphin and α-melanocyte stimulating (α-MSH)

hormone in the MBH of alcohol-treated *Per2* mutant and wild type mice at postnatal day 7 and 90. Postnatal ethanol exposures suppressed *Pomc* and metabolic genes and *Pomc*-derived products β-endorphin and α-MSH in the MBH for a prolonged period of time. *Per2* mutation prevented these effects of postnatal ethanol on *Pomc* and metabolic genes. Furthermore, in order to determine the effect of *Per2* gene deletion on postnatal alcohol programming of the circadian expression of metabolic genes in *Pomc* neurons, we created an *Egfp- Pomc -Per2* mutant mice and employed these mice to determine gene expression in Laser captured isolated *Pomc* expressing neurons. We found that postnatal ethanol exposures permanently altered circadian expression of *Pomc* and metabolic genes in *Pomc* neurons. *Per*2 mutation prevented the postnatal ethanol-induced alteration of metabolic genes in *Pomc* neurons. Together, these data suggest that early life exposure to ethanol alters metabolic sensing in *Pomc* expressing neurons possibly via regulation of *Per2* gene.

DEDICATION

I'd like to dedicate this work to my family who has supported me in everything I've accomplished, especially my parents, their moral and family support always gave me power to continue and surpass any obstacle I encountered throughout my PhD training; My in-law's for their family support without their help this degree will not have been possible; My husband, his unlimited love, support and understanding made this journey a success; My son, the most important person in my life, I thank him for understanding the sleepless nights in the computer, for giving me juice and a cookie to keep me awake.

Lastly, I will like to also dedicate this thesis to Dr. D'mitry Govorko, who was a good friend and scientist.

"Most people say that it is the intellect, which makes a great scientist. They are wrong: it is character."

Albert Einstein

"It is the supreme art of the teacher to awaken joy in creative expression and knowledge."

Albert Einstein

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TABLE OF CONTENTS

LIST OF FIGURES

SUPPLEMENTARY DATA

LIST OF ABBREVIATIONS

ACTH, Adrenocorticotropic hormone

Arc, arcuate nucleus

Asb4, ankyrin repeat and SOCS box containing 4

β-Endo, βeta-endorphin

Bmal1, brain and muscle ARNT-Like 1 gene

CCG, clock-controlled gene

CDC, center for disease control

Clock, Circadian Locomotor Output Cycles Kaput

CNS, central nervous system

Cry, cryptochrome gene family

CRH, Corticotropin-releasing hormone

CT, circadian time

DD, constant dark conditions

ELISA, enzyme-linked immunosorbent asay

FAS, fetal alcohol syndrome

FASD, fetal alcohol spectrum disorders

GAPDH, glyceraldehyde-3-phosphate dehydrogenase

h, hour

HPA, hypothalamic-pituitary-adrenal axis

IUGR, intrauterine growth retardation

IOM, institute of medicine of the national academy of sciences

LD, constant light/dark conditions

αMSH, alpha-melanocortin stimulating hormone

NIH, National Institute of Health

PAE, prenatal ethanol exposure

PD , postnatal day

Per, period gene family

POMC, proopiomelanocortin

Pgc1α (aka PPARGC1A), Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PVN, paraventricular nucleus of the hypothalamus

SCN, suprachiasmatic nucleus

siRNA, small interfering RNA

Sirt1, sirtuin 1

Stat3, Signal transducer and activator of transcription 3

WT, wild type

ZT, zeitgeber time

CHAPTER 1

Review of the literature

Overview of Fetal Alcohol Syndrome

The CDC has reported analysis from a 2002 study, indicating that approximately 7.6 % of pregnant women in the United States used alcohol and 1.4 % of these women engage in binge drinking (CDC 2012) [1]. Despite the targeted educational awareness efforts, pregnant women continue to drink [2, 3]. Even though, this study only examines the drinking rate of pregnant women, these women can have a higher rate to have a child with developmental problems.

It has been shown that alcohol exposure during pregnancy can lead to fetal alcohol syndrome (FAS); which is a leading non-genetic cause for mental retardation in the Western world [3]. This syndrome is characterized by the following: growth deficiency. Central nervous system disorders and a pattern of distinguishable facial characteristic in those affected [4-6]. In the United States during the 1980s and 1990s, the prevalence of FAS was 0.5 to 2 cases every 1,000 births [4, 7]; it is believed that this numbers have increased. Nevertheless, FAS represents only the extreme end of the spectrum of effects that can occur following prenatal exposure to alcohol [4, 5]. There are cases in which the severity is not the full spectrum of the syndrome, but a range of effects (that is observed in FAS patients) that occur in an individual whose mother drank alcohol during pregnancy this is termed as fetal alcohol spectrum disorders (FASD).

FASD effects can be physical, mental, or behavioral with possible lifelong implications [8]. The estimate prevalence of FASD is about 1 per 100 (1% of live births)

[7]. Therefore alcohol used during pregnancy can have devastating effects on the developing embryo.

Based on the Institute of Medicine of the national academy of sciences (IOM), the following criteria's are used to diagnose FAS. The IOM has categorized FAS into two spectra, FAS based on maternal consumption of alcohol during pregnancy, or FAS without confirmed maternal exposure [2, 4, 5].

The FAS characteristics are the following: facial anomalies are observed in the offspring, such as short palpebral fissures, and abnormalities of the premaxillary zone (e.g. flat upper lip, flattened philtrum, flat midface); growth deficiencies are observed (low birth weight, lack of weight gain over time) with disproportional low weight to height ratio; and distinguishable neurodevelopmental abnormalities are observed in the neonate, including a small head size at birth, impaired fine motor skills, neurosensory hearing loss, poor tandem gait, and poor eye-hand coordination [2, 4, 5].

Alcohol is known to be a xenobiotic substance; it interacts with tissues in a multitude of ways its effects can be long-term. Alcohol exposure is an example of an insult that is referred as fetal or early programming, which is the concept that early environmental or non-genetic factors, including pre- or postnatal exposure to drugs or other toxic agents, can permanently organize or imprint physiological and behavioral systems and increase vulnerability to illnesses or disorders later in life [9-13]. Whether ethanol-induced HPA axis or metabolic imbalances that contribute to the etiology of FAS or FASD is unknown [14].

The diverse doses of alcohol and the timing of exposure during pregnancy seem to result in diverse phenotypes as it observed in FASD patients. Thus, the amount of

alcohol that reaches the developing embryo or fetus at a particular developmental phase is critical [8]. The use of fetal alcohol animal models has helped in depicting altered mechanisms by ethanol. The full gestation period (prenatal life) in rodents is equivalent to the first and second trimesters in humans, while postnatal day one (PD1) to PD10 corresponds roughly to the third trimester in humans [15-22]. This suggests that exposure to alcohol during pre-and postnatal periods is expected to produce similar deficits as seen in offspring of human mothers who abuse alcohol during pregnancy [22]; making the rodent animal model a good model system to study developmental effects of alcohol.

Anatomical analysis has demonstrated that prenatal alcohol exposure during the first trimester interferes with the migration, proliferation, and organization of brain cells [8, 15-17], whereas alcohol consumption in the third trimester are highly related to damage to the cerebellum, hippocampus and prefrontal cortex; suggesting that alcohol impairs different regions of the central nervous system (CNS) depending on the time of its exposure during pregnancy [8, 18-21].

In the last two decades, studies have revealed that ethanol not only induces the facial anomalies, neurodevelopmental disorders or altered motor skills as described before; but it also affects physiological mechanisms that are important in maintaining physiological responses (such as stress response homeostasis, metabolic signaling and circadian rhythms).

Fetal Alcohol Syndrome and the stress response

Secondary disabilities have been associated with FAS and FASD patients such as mental illness, depression, and anxiety disorders [9, 23-26]. Interestingly, all these diseases are associated with the hypothalamic-pituitary-adrenal (HPA) axis [27, 28].

Several studies have shown that prenatal alcohol exposure may alter hormonal rhythmicity or hormonal output. For example, Handa et al., demonstrated that prenatal ethanol exposure modify the circadian regulation of core body temperature and corticosterone release in anticipation to feeding [29].

Studies by Weinberg and others have shown that prenatal alcohol exposure results in HPA hyperactivity and altered regulation of the HPA comparable to the model system for depression. Their data revealed that prenatal alcohol exposed animals exhibit hyper-responsiveness to stressors, show increase HPA drive, and demonstrate deficits in the HPA feedback regulation [9, 14, 28]. These data support the idea that prenatal exposure to ethanol programs the HPA axis, sensitizing the organism to subsequent stressful experiences and thereby increasing the propensity to develop depression in adulthood [9, 14, 27, 28].

Fetal Alcohol Syndrome and metabolic signaling

Studies have shown that fetal alcohol exposure alters metabolic signaling pathways, predominantly the gluconeogenesis process [30]. It is known that approximately 60% of fetal glucose is derived from the maternal source and the remainder is produce by the fetus itself [31, 32]. In addition, it is known that fetal hepatic glycogen accumulates during late gestation and its synthesis parallels increase in the activities of enzymes involve in glycogen metabolism or its breakdown to glucose. Singh et al., demonstrated that ethanol in utero manifested a hypoglycemia state, low fetal weight, low fetal liver weight, low liver glycogen level, low glycogen synthase activity and low phosphorylase activity in compare to control rat pups group [33]. This data

suggests that ethanol may inhibit the fetal glucose output by altering the glycogen breakdown to glucose. How does this happen?

This can be as a result of two possibilities: 1. Ethanol inhibits the glucose output by either inhibiting the expression of key gluconeogenic enzymes; or 2. Ethanol alters the redox state [33, 34]. Expanding this notion further, Tanaka et al. demonstrated that administration of glucose to pregnant rats during late gestation counteracted the fetal brain weight decrement associated with fetal alcohol exposures in aimal model system [35]. All this studies support the idea that prenatal alcohol exposure disrupts gluconeogenesis during development and predisposes the ethanol-exposed neonates to a hypoglycemic state.

The next question should be “Does the effect of alcohol on gluconeogenesis last till adulthood?” The answer to this question still remains to be elucidated, but early works have suggested that these fetal effects of alcohol are sustained in the adult [30, 36, 37]. Studies by Nyomba have led to believe that the effect of alcohol exposure during pregnancy not only is associated with fetal growth restriction but also it may destine the offspring to insulin resistance later in life. His research focused on whether altered regulation of gluconeogenesis and adipose hormones may be caused by alcohol exposure during pregnancy [38]. Newborn rats from dams that had been given ethanol during pregnancy were studied and the adiponectin mRNA levels were measured in subcutaneous fat by RT-PCR, serum adiponectin were measured by RIA, hepatic expression of gluconeogenic enzymes and that of the transcription factor peroxisome proliferator-activated receptor-coactivator (PGC)-1, which promotes gluconeogenesis were examined by RT-PCR. Their results demonstrated that ethanol exposed offsprings

had delayed hypoglycemic response to insulin but normal adiponectin mRNA. The inhibitory response of the gluconeogenic enzyme phosphoenol- pyruvate carboxykinase (PEPCK) and PGC-1 mRNAs to insulin was blunted in ethanol-exposed offspring compared with controls. The authors concluded that intrauterine ethanol exposure causes insulin resistance of genes for PGC-1 and PEPCK early in life [38].

Nyomba had demonstrated that rat offspring exposed to ethanol during pregnancy are insulin resistant, but it is unknown whether they have increased gluconeogenesis which is expected based on their previous findings. Therefore, Nyomba et al., continued their study by measuring blood glucose, liver gluconeogenic genes, proteins, and enzyme activities before and after insulin administration in juvenile and adult rats prenatally exposed to ethanol. The results showed that in juvenile rats, basal glucose; PGC-1a protein and mRNA; and PECK activity, protein, and mRNA were similar between groups. It is only after insulin injection that these parameters failed to decrease in ethanol exposed rats, However in control rats glucose decreased by 30% and gluconeogenic enzymes, proteins, and mRNAs decreased by 50-70% [38].

In adult offspring, basal hepatic PGC-1a protein and mRNA levels were 40-80% higher in ethanol rats than in controls. Similarly, basal PEPCK activity, protein, and mRNA were approximately 1.8-fold greater in ethanol-exposed rats than in controls. These parameters decreased by approximately 50% after insulin injection in control rats, but they remained unchanged in ethanol rats. After insulin injection in the adult rats, glucose decreased by 60% in controls but did not decrease significantly in ethanol rats. A subset of adult ethanol rats had fasting hyperglycemia and an exaggerated glycemic response to pyruvate compared with controls [39]. The authors concluded that after

prenatal EtOH exposure, the expression of gluconeogenic genes are altered in adult rat offspring and an insulin resistant phenotype develops in both juvenile and adult rats, it is believed that these alterations persist through adulthood and may contribute to the pathogenesis of Type 2 diabetes after exposure to ethanol in utero. Interestingly, Nyomba's lab concluded that perhaps ethanol disrupts the proopiomelanocortin system resulting in damage to the melanocortin system, leading to a metabolic disorder state [39, 40].

The developmental exposure to ethanol produce diverse alterations to endocrine signals, but one thing can be elucidated from these findings is that alcohol exposure is somehow altering body tissues important in mediating metabolic signaling processes.

Fetal Alcohol Syndrome and circadian rhythms

Some investigators have speculated that the body's internal clock, which is located in the suprachiasmatic nuclei (SCN) in the hypothalamus, may also be affected by developmental alcohol exposure. It is believed that alcohol-induced damage to the SCN cells and their function could result in disturbances of the circadian timekeeping function, and these disturbances might contribute to the behavioral impairments and affective disorders observed in people prenatally exposed to alcohol [41-45].

Studies conducted in rats suggest that developmental alcohol exposure may indeed interfere with circadian clock function. Compiled data suggests that alcohol shortens the circadian sleep-wake cycle leading to the development of seasonal and non-seasonal mood disorders; it changes the release of certain neuropeptides by SCN cells; it leads to a long lasting effect on the light responsiveness of the deep body temperature circadian rhythm, it interferes with the light sensitivity of the suprachiasmatic nucleus

(SCN) by suppressing brain derived neurotrophic factor (BDNF) expression; and it decreases the number of serotonin neurons in the brainstem [44].

In addition, Sakata-Haga et al. demonstrated that ethanol exposure during the third trimester disrupts the ability to synchronize circadian rhythm to light cues. Disruptions in circadian regulation may cause abnormal behavioral rhythmicity, such as disrupted sleep and feeding patterns, as seen in individuals prenatally exposed to ethanol [46]. Similarly, this lab continue the study by analyzing the effects of pre- or postnatal exposure to ethanol on the circadian rhythm in adulthood by measuring deep body temperature and wheel running activity in rats. After a phase delay in the light/dark cycle, ethanol-exposed rats took longer than control rats to resynchronize to the new light/dark cycle [47]. These results suggested that both pre- and postnatal ethanol exposure impair the development of the circadian clock response to light cue. The authors speculated that due to the abnormal development of the circadian clock system this might contribute to the neuropsychiatric symptoms seen in FASD, it is believed that normalizing the disturbed rhythm improves the symptoms [46, 47]. However, Sakata-Haga and others suggests that the mechanisms of dysfunction and potential interventions for disturbance of circadian clock system still remain to be elucidated [46-49].

Handa et al., examined the potential functional consequences of ethanol's interference with hypothalamic differentiation, they studied the long-term effects of prenatal ethanol exposure (PAE) on basal circadian rhythms of core body temperature, heart rate and surge in corticosterone. The results showed that the core body temperature circadian rhythm were altered, no significant effects of (PAE) were observed on basal heart rate rhythm at any age, and the diurnal rise in corticosterone secretion was blunted

and prolonged in 6-month-old PAE males compared to controls [29]. The authors suggested that exposure to ethanol during the period of hypothalamic development can alter the long-term regulation of circadian rhythms in specific physiological systems [29].

Furthermore, clinical studies revealed the alcohol consumption during pregnancy has a long lasting effect on the sleep cycle in fetal alcohol exposed infants. Troese et al., studied the relationship between prenatal alcohol exposure, sleep, arousal and sleep-related spontaneous motor movements in early infancy. The results showed that prenatal alcohol exposure disrupts postnatal sleep organization and suppresses spontaneous movements during sleep, and increased sleep fragmentation that promotes sleep deprivation [41].

In addition, Pesonen et al., examined whether small body size at birth and prenatal tobacco or alcohol exposure predict poor sleep and more sleep disturbances in children. Their results demonstrated that children exposed prenatally to alcohol had a 2.9-fold and 3.6-fold increased risk for having short sleep and low sleep efficiency [42]. The authors concluded that poor sleep in children may have prenatal origins, the possible mechanisms for this include alcohol consumption during pregnancy and other conditions associated with small body size at birth [42].

Finally, Wengel et al., aimed to characterize sleep in FASD and describe the impact of sensory processing difficulties on sleep patterns in children with FASD. Their results showed that children with FASD have significantly more sleep disturbances than typically developing children, including increased bedtime resistance, shortened sleep duration, increased sleep anxiety, and increased night awakenings and parasomnias (partial arousals during the transitions between wakefulness and NREM sleep, or

wakefulness and REM sleep) [43]. The authors suggested that children with FASD should be screened for sleep-related disorders [43].

These studies demonstrated that sleep deficits are widespread in children with FASD, suggesting that ethanol disturb the body's circadian clock in maintaining homeostasis.

Our lab's scientific contributions to fetal alcohol syndrome

Dr. Dipak Sarkar's lab has contributed significantly to the study of alcohol and fetal alcohol syndrome. Early in the 2004, his lab identified the sensitivity of the circadian clock system and its opioidergic system to chronic alcohol exposure. The data showed that chronic alcohol exposure altered both the circadian rhythm of pro-opiomelanocortin (POMC) mRNA expression and the circadian expression of the clock governing rat *Period* genes (rPeriod1 mRNA and rPeriod2 mRNA) in the arcuate nucleus of the hypothalamus. Similarly, the circadian expressions of the clock rat period genes (rPeriod2 mRNA and rPeriod3 mRNA) were altered in the SCN of the hypothalamus [50]. This data suggested that ethanol administration disrupted the internal clock of beta-endorphin neurons in the arcuate nucleus and SCN of the hypothalamus [50]. This finding led to further investigations relating to the effects of ethanol at the developmental period, mainly focusing on the POMC gene expression and its biological peptides.

Since studies have suggested that sleep-wake disturbances and altered stress responses were observed in FAS patients, our lab decided to investigate whether fetal ethanol exposure during the gestational days 10-21 affects the circadian function of the stress-axis regulatory beta-endorphin neurons in the hypothalamus. The results demonstrated an altered circadian expression of the clock governing Period genes (rPer1,

rPer2, and rPer3) and an abnormal POMC gene profile in the arcuate nucleus of the hypothalamus; and an altered rPer1, rPer2 gene profile in the SCN of the hypothalamus during the adult period [51]. For the first time, our lab showed that fetal exposure to ethanol significantly alters the clock mechanisms governing the circadian function of beta-endorphin neurons.

It is known that POMC expressing neurons in the arcuate nucleus of the hypothalamus secrete β-Endorphin, a biological peptide that controls a variety of physiological functions including the feedback regulation of the CRH neuronal activity in the paraventricular nucleus of the hypothalamus.

Our lab proposed, "If prenatal alcohol alters the expression of POMC gene, then there is a possibility that prenatal ethanol induces β-endorphin neuronal death and alters their influence on CRH neurons in vivo." Using a binge like ethanol exposure model, they demonstrated the following: (1) An increase in the number of apoptotic β-Endorphin neurons in the arcuate nucleus of the hypothalamus (2) Reduction in POMC and adenylyl cyclases mRNA (3) Increased expression of several TGF-β1- linked apoptotic genes (4) Reduction in the number of β-Endorphin terminals in the paraventricular nuclei of the hypothalamus (5) Increased response of the hypothalamic CRH mRNA to lipopolysaccharide (LPS) challenge (6) Showed inability to respond to exogenous β-Endorphin to alter the LPS induced CRH mRNA levels [52]. This data finding and others from this lab proposed that ethanol hinders the responsiveness of beta-EP-secreting neurons to CRH and VIP, leading to alter HPA response [53, 54].

Subsequently, studies have suggested that disruption of circadian clock may inhibit an immune response against infection and cancer. Thus, our lab wanted to

investigate whether natural killer (NK) cells were rhythmic and whether prenatal ethanol exposure influences this rhythmicity. The results showed that adult rats exposed to ethanol during their fetal life showed a significant alteration in the physiological rhythms of granzyme B and IFN-γ that was associated with decreased NK cell cytotoxic activity [55]. The authors concluded that fetal ethanol exposure causes a permanent alteration of specific immune rhythms that may in part underlie the immune impairment observed in FAS children.

In note with this finding on immune impairment, Polanco et al. showed that alcohol exposure in utero increases susceptibility to mammary tumorigenesis in adulthood and alterations in the IGF and E2 systems may play a role in the underlying mechanism [56]. Interestingly, our lab demonstrated that beta-endorphin neurons had a protective role in mammary carcinoma; Sarkar's lab developed a technique to generate and isolate beta-endorphin neurons via in vitro differentiation from fetal neuronal stem cells. Then these cells were transplanted into the hypothalamus of rats subjected to breast carcinogenesis. The results showed that rats containing the in-vitro beta-endorphin neurons displayed a reduction in mammary tumor incidence in compare to the control groups [57]. The author's postulated that beta-endorphin neurons served as a shield against the growth and metastasis of mammary tumor cells by altering the autonomic nervous system (ANS) activities that enhance innate immune function.

Hence, Sarkar's lab has contributed to the notion that alcohol impairs both the circadian clock and the opioidergic system leading to disturbances in the immune system. The next question may be what are the other effects of gestational alcohol exposure to the opioidergic system and metabolic signaling?

Structure of the pro-opiomelanocortin (POMC) gene

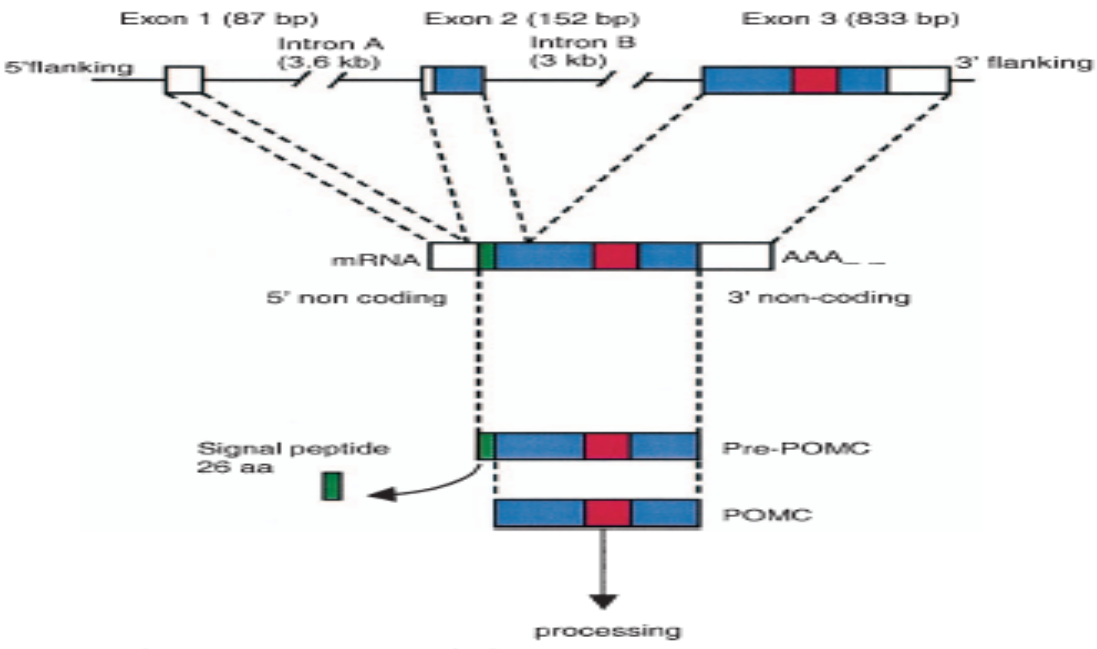


Figure 1: Structure of the pro-opiomelanocortin (POMC) gene processing. Reformatted: Newell-Price 2003 (69).

In humans, the POMC gene resides in chromosome 2p23 [58, 59]. This gene was discovered four decades ago (in 1981) [59]. In 1982, the POMC gene regulatory regions were identified [60, 61], and the year after Takahashi et al, reported its complete gene sequence [62]. This gene consists of three exons, intermixed with large introns (the first of which is un-translated), and it spans about 7665 bp [63]. Furthermore, It is exon 2 and 3 that encompasses the protein coding region [64].

The characteristics of the three exons are the following: Exon 1 encompasses 87 bp of untranslated sequence. This exon is called the leader sequence because it binds to ribosomes to facilitate the onset of translation. Exon2 contains the initiation sequence that is part of the 5' untranslated mRNA, which is 152 bp long. This sequence codes for the 26 amino acid signal sequence required for transport across the rough endoplasmic

reticulum, and the first amino acids of the N-terminal region. Exon 3 is composed of 833 bp. This exon encodes for the majority of the translated mRNA and for the 3' untranslated region [61-63].

The POMC gene is conserved across all species, hence highlighting the importance of the gene and its peptide products in maintaining organism's physiological processes [65, 66].

Mammals transcribe only one POMC gene per haploid genome [67], while the mouse genome contains two non-allelic POMC α- and β-genes [68-71]. The mouse POMC α-gene is transcribed in both the pituitary and brain. The mouse POMC is located on chromosome 12 and it shares high homology with human, bovine, and rat POMC genes [70]. The POMC β-gene has 90% amino acid sequence homology (predicted from cDNA sequence) with α-POMC gene [71]; However the POMC β-gene is not expressed in the pituitary. In addition, the POMC β-gene has the characteristics of a pseudogene, and is found on chromosome 19 [71]. Lower vertebrates including lamprey, fish, and frogs also express two forms of POMC gene that have different degree of homology depending on the species [72, 73]. This matter may be an evolutionary factor.

The POMC gene is expressed in a ubiquitous manner. This gene is found at significant levels in tissues such as the pituitary, brain, placenta, and epithelium, which are derived from the ectodermal neural plate [59, 74-77]. In the brain, the POMC gene is expressed in the arcuate nucleus of the hypothalamus, amygdala, the cortex, and the nucleus tractus solitaries of the brainstem [78, 79]. In the pituitary, the POMC gene is expressed predominantly in the anterior pituitary where it comprises 30 % of all mRNA [80], thus the POMC mRNA concentration is one to two orders of magnitude higher in

pituitary than in brain [81, 82]. In contrast, the POMC gene is expressed in minimal quantities in the following organs: testis, ovary, duodenum, liver, kidney, adrenal medulla, lung, thymus, heart, lymphocytes and in some tumors [83-93]. The regulation of this gene varies depending on the tissue.

POMC mRNA transcripts

The POMC gene contains three different promoters, which differ in their tissue distribution of expression. For example in the anterior pituitary and hypothalamus, the POMC transcription starts from the 5' region upstream of exon 1 and the product is cleaved to form the 1150 nt mRNA transcript. During translation, this transcript forms the prePOMC peptide [63]. In peripheral tissues such as: testes, ovary, placenta, lymphoid cells, adrenal medulla, and tumor tissue, low amounts of mRNA (800 nt in length) is expressed from a promoter within intron B, localized upstream of exon 3. The transcription product is a shorter mRNA transcript that lacks the signal peptide, thus the translated protein is not secreted. The function of this transcript is unclear [63, 81, 85, 86, 90, 94-100]. Tumors such as small cell lung cancer or carcinoid tumors of bronchial origin, transcribe a 5' extended mRNA that is 1350 nt long. This transcript is translated eventually to the same size POMC peptide (1150 nt) expressed in the pituitary, because the transcript has only one translation initiation site. Although the major transcript in the pituitary is 1150 nt in length, very small quantities of the 1350 nt transcript are also present [99, 101-104].

Regulatory mechanisms for gene transcription in the pituitary and hypothalamus

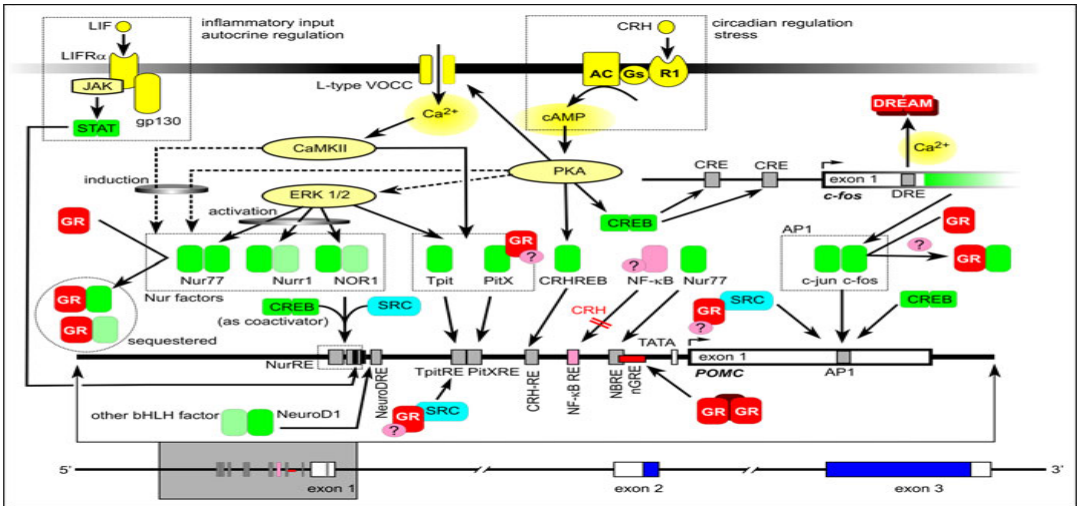


Figure 2: Reformatted: Jenks 2009 (71).

Figure 2. Regulatory mechanism for POMC gene transcription. Reformatted: Jenks 2009 (71).

I. The pituitary

In this location, the POMC gene has two different pituitary cell lineages, the corticotropes and melanotropes, its transcriptional regulation and processing of the POMC polypeptide are highly controlled by transcription factors sites that are either localized nearby the promoter site, upstream of the promoter or by upstream enhancers. The POMC regulatory elements found adjacent to the promoter site serves to mediate its transcription and to some extent function similarly in all tissues studied [105]. The elements found near the promoter site are: the homeobox transcription factors site for Pitx and Tpit; the Stat3; NGF1-B (Nur77), CRH-RE, NF-κB, NF-κBRE and SF1 [64, 106-109]. The Pitx and Tpit transcription factors are required for terminal differentiation of the corticotrope and melanotrope lineages and for cell-specific transcription of the POMC gene [108, 109]; the orphan receptor NGF1-B controls pituitary POMC transcription; this is CRH mediated in part by cAMP response element binding protein

(CREB), it upregulates NGF1-B expression resulting in increased POMC transcription. In contrast, glucocorticoids mediate their inhibitory effect on POMC transcription by interacting with glucocorticoid receptor (GR) with DNA-bound NGF1-B [110, 111]. The CRH-RE binding site has a wide tissue distribution [64]. The presence of NF-κBRE binding site in the POMC promoter is unknown; however activation of the NF-κB binding site inhibits the expression of the POMC gene.

The POMC regulatory elements sites upstream of the promoter site are: a second Lif activated Stat3 binding site, a Dex-dependent glucocorticoid receptor (GR) site, a CRH activated Nurr1 (a nuclear receptor of the Nur subfamily -NBRE), E boxes that are binding sites for transcription factors of the bHLH family such as NeuroD1, which are expressed in corticotrophs but not melanotrophs [107].

The 7 - kb enhancer is dependent on the palindromic TpitREpal. The TpitREpal is the first reported natural palindromic response element for transcription factors of the T box family. The 7 - kb enhancer contains putative binding sites for all the transcription factors of the POMC promoter except the Pitx binding site. [108]. The enhancer activity is dependent on Tpit, therefore it is suggestive that this enhancer may function as a lineage restricted amplification factor, whereas the promoter driven by Pitx served as the initial activation of the POMC locus [107].

The POMC regulation mechanism is not the same in neuronal POMC or other tissues. There are some tissue-dependent differences in the control of POMC expression. Thus, a specific POMC gene expression in the pituitary depends on two factors: a combination of binding sites for these cell restricted factors, and the expression of ubiquitous transcription factors that mediate the action of signaling pathways. These

factors must be present within the regulatory sequences, promoters and enhancers sites of the POMC gene, in order to mediate a direct pituitary POMC gene expression [107].

II. The hypothalamus

There are approximately 3,000 POMC –expressing neurons in the arcuate nucleus of the mouse hypothalamus [112, 113]. The POMC gene contains a 400-bp proximal POMC promoter, capable of directing reporter gene expression in corticotrophs and melanotrophs in the pituitary. However, this promoter is incapable of directing this expression to POMC hypothalamic neuron [64, 114]. Studies revealed distant regulatory elements served to modulate hypothalamic regulation of POMC transcription [105]. These regulatory elements are called neuronal POMC enhancer (nPE1) and (nPE2). The characteristics of these enhancers are the following: (1) the distal genomic region containing nPE1 and nPE2 is necessary to direct neuronal expression of POMC in the arcuate nucleus. (2) Either nPE1 or nPE2 can permit proper reporter expression in POMC arcuate neurons; however simultaneous elimination of nPE1 and nPE2 terminates expression in POMC neurons. (3) nPE1 or nPE2 nucleotide sequences and genomic organization are highly conserved among mammals but not between mammals and birds, amphibians, or fishes. (4) The enhancer activity of mouse and human genomic fragments containing nPE1 or nPE2 is functionally conserved. (5) POMC expression in the brain and pituitary gland is controlled by different and independent sets of enhancers. (6) A limited number of conserved and aligned putative transcription factor binding sites are present in these enhancers. nPE1 encompasses DNA sequences for STAT3, POU-domain proteins Brn 4.0 , OCT-1, and two separate conserved CREB –like sites; whereas nPE2 comprises homeodomain factors such as Nkx6.1 and Brn2.0 [64, 105].

Due to the different transcription factor binding sites found in each enhancer, it is possible that each nPE enhancer plays a role in mediating POMC transcription. For instance, leptin receptors localized in POMC arcuate neurons signal via phosphorylated STAT3 and deficits in this signal transduction pathway within these neurons correlate with very low levels of POMC mRNA [105, 115, 116]. Furthermore, heterologous cell line showed that STAT3 might increase POMC transcription by interacting with a non-canonical STAT3 binding site located immediately upstream of the TATAA box [117]. nPE1 may be a key player for POMC transcription through this pathway. Adjacent to the nPE1 enhancer, a consensus sequence for NERF/Elf-2, were found. This sequence is a member of the Ets transcription factor family. Interestingly, Both POU domain and Ets factors have been shown to form heterodimers to activate gene expression [64, 105]. In the case for nPE2 enhancer, there are two overlapping canonical sequences for COUP and ERRα, the binding transcription factors for this sites belong to the orphan nuclear receptor family. The presence of these binding sites suggest that a physical interaction with a homeodomian factor may occur to repress POMC expression, similar mechanism has been observed in the pituitary [105, 118]. In addition, an additional ERRα site has been located downstream at the 3' end of nPE2. It has been suggestive the purpose of this ERRα site is to control energy homeostasis by regulating food intake in collaboration with leptin and NPY activation of POMC neurons [64, 105].

The complex promoter/ enhancer structure of the POMC gene enables both the pituitary and hypothalamus to integrate a different set of inputs. This leads to the different ratios of biological products yield by the POMC gene at specific tissues.

The pro-opiomelanocortin (POMC) polypeptide

At the molecular level, The POMC encodes a precursor polypeptide (pro-hormone) that contains 241 amino acid residues and it weighs 32 kDa [119]. This polypeptide is cleaved to produce the following biologically active peptides such as opioid peptide β-endorphin, Adrenocorticotropic hormone **(**ACTH) and α-melanocyte stimulating hormone (αMSH) [65, 66, 120] . The degree of expression of these biological active peptides differs in location due to the tissue specific post-translational processing of the POMC polypeptide.

In the anterior pituitary, POMC is processed predominantly to ACTH and β-lipotropin. However in the intermediate lobe and hypothalamus, further processing continues to β-EP, γ-lipotropin, α-MSH and corticotropin-like intermediate-lobe peptide [121, 122].

The POMC polypeptide processing in the Nervous Tissue

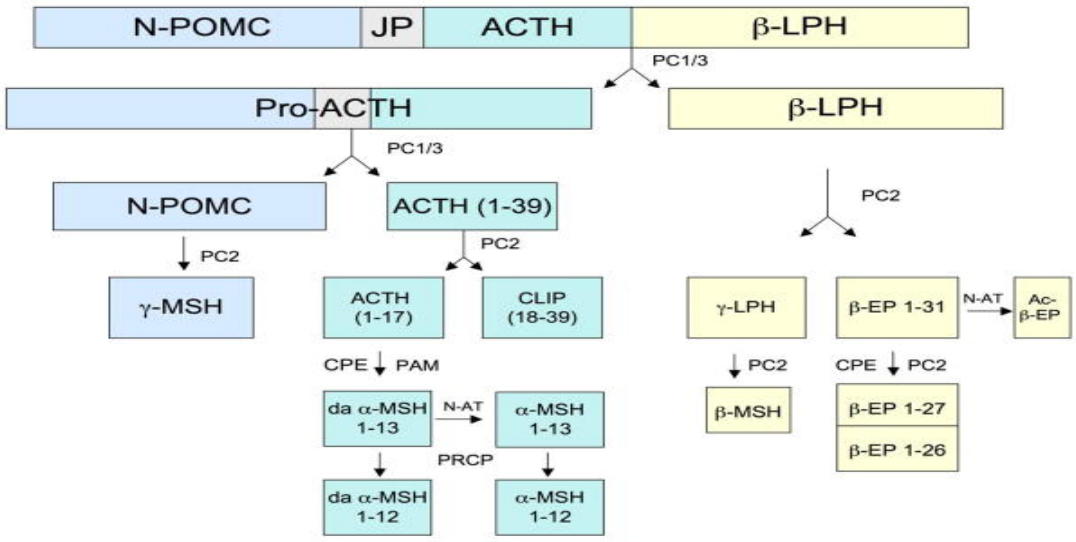


Figure 3. The POMC polypeptide processing. Reformatted from Wardlaw 2011 (145).

I. Pituitary

In the pituitary, the POMC expression is under the main positive regulatory control of CRH, and negatively regulated by glucocorticoids produced by the adrenal gland, this mechanism is known as the negative feedback loop [110, 111]. The posttranslational processing of the POMC prohormone is tissue specific. This process begins when the polypeptide passes through the Golgi bodies and it is in there that the signal peptide sequence directs it into the secretory granules, where post-translational processing yields the different biologically active peptides that are induce by successive, cell specific, enzymatic modifications [120, 123]. This results in the production of different POMC peptides (as mentioned above) by different cell types and therefore provides latitude for the control of multiple physiological functions by the same prohormone. Processing is performed at basic cleavage sites by the cathepsin L pathway [124] or prohormone convertases called PC1/3 and PC2 [125-128].

In the anterior pituitary, PC1 alone is expressed in corticotrophs cells and it cleaves POMC, producing the terminal peptide, joining peptide, ACTH, β and γ-lipotropin (LPH) and β-endorphin [58, 128]. However, in the intermediate lobe of the pituitary, PC1, PC2, carboxypeptidase E (CPE), amidating and N-acetylating enzymes are generated in melanotrophs to produce αMSH and β-endorphin [88, 128, 129] . It is PC2 that cleaves the first 14 amino acids of ACTH to generate $ACTH^{(1-14)}$-OH. Then, this molecule after COOH-terminal amidation, produces N-acetylated forms of α-MSH, a step required for the biological activity of MSH [128, 130, 131]. Similarly, PC2 cleaves β-LPH to produce β-MSH and β-endorphin. Finally, N-acetylation occurs on the majority of the β-endorphin molecules resulting in the destruction of its opiate activity [128, 132].

In conclusion, the POMC polypeptide from the anterior pituitary is processed to ACTH, β-lipotropin (LPH) and a 16 kDa N-terminal fragment, among these products; it is ACTH that predominantly aids to maintain the adrenocortical function.

II. In the hypothalamus

The hypothalamus is the master regulator of energy intake, energy expenditure, body weight balance, HPA axis, body temperature, sleep, and circadian cycles [133-136]. The hypothalamus contains a number of small nuclei with a variety of functions, particularly the arcuate nucleus. In the brain, POMC is predominantly expressed in two discrete regions, the arcuate nucleus (ARC) of the hypothalamus and the commissural nucleus tractus solitarius (NTS) of the brainstem [119]. Interestingly, there are fewer POMC expressing cells in the NTS than those in the ARC [119, 120], suggesting that the major contribution of neuronal POMC comes from the ARC instead of the NTS brain region. It is for this reason that the latter information will focus on the POMC expression from the arcuate nucleus.

The posttranslational processing of the POMC prohormone is tissue specific. This process begins at the Golgi bodies; it is the signal peptide (N-terminal sequence) and the membrane carboxypeptidase E (CPE) that directs the POMC polypeptide into the secretory granules. The CPE binds to the N-terminal POMC sequence and serves as sorting receptor [137]. During this trafficking process, the POMC polypeptide is proteolytically cleaved into different biologically active peptides via successive, cell specific, enzymatic modifications [120, 123, 138]. The differential expression of prohormone convertases (PCs) in various tissues leads to tissue specific posttranslational processing of POMC [122, 139]. Functional active peptides are produced by

endoproteolytic cleavage at adjacent pairs of basic amino acids by the prohormone convertases, PC1/3 and PC2 [140].

Both in the hypothalamus and in the intermediate lobe of the pituitary, POMC is more extensively processed: ACTH is further processed to produce α-MSH and corticotropin-like-intermediate lobe peptide (CLIP); β-LPH is processed to γ-LPH and β-EP; N-terminal POMC is processed to γ_3-MSH [121, 141]. In the human, γ-LPH can be further processed to β-MSH [138, 142].

A scheme of POMC processing in the hypothalamus is depicted in Figure 3. POMC is initially cleaved by PC1/3 to yield pro-ACTH and β-LPH. Pro-ACTH is then cleaved by PC1/3 to ACTH and N-terminal POMC. Further processing by PC2 yields ACTH $_{1–17}$ and CLIP as well as γ-LPH and β-EP $_{1–31}$. Another processing enzyme, carboxypeptidase E (CPE), removes the C-terminal basic amino acid residues from ACTH $_{1–17}$ to form ACTH $_{1–13}$ which is then amidated by the enzyme peptidyl α-amidating monooxygenase (PAM) to generate desacetyl α-MSH [138]. Desacetyl α-MSH can then be acetylated by an N-acetyltransferase to form α-MSH. Desacetyl α-MSH is the predominant form of α-MSH in rodent hypothalamic extracts, whereas N-acetyl α-MSH is the predominant form in the intermediate lobe of the pituitary [143]. Recently a new processing enzyme, prolylcarboxypeptidase (PRCP) has been identified that is responsible for inactivation of α-MSH by removal of the C-terminal valine [144]. Processing of β-LPH by PC2 yields γ-LPH and β-EP $_{1–31}$, one of the endogenous opioid peptides. β-EP can also be acetylated resulting in loss of opioid activity. As with α-MSH, acetylation of β-EP occurs to a large extent in the intermediate lobe of the pituitary but not in the hypothalamus. Although there is little acetylation of β-EP in the hypothalamus,

β-EP $_{1–31}$ can be cleaved by PC2 and CPE to β-EP $_{1–27}$ and $_{1–26}$, which have markedly reduced opioid activity [138, 145, 146].

The role of POMC peptide products in stress hormonal output

I. The hypothalamus role

In the hypothalamus, the paraventricular nucleus (PVN) is the site for integration of environmental and interoceptive triggers of stress [147]. PVN corticotropin releasing hormone (CRH) neurons are neuronal targets implicated in the anorectic and catabolic effects of melanocortins [147]. It is PVN CRH neurons that release CRH from terminals in the median eminence to the pituitary. It is the portal venous plexus that connects the CNS with the pituitary gland and where CRH enters and is destined to pituitary cells. In the pituitary, CRH activates CRH-R1 receptor located on both anterior lobe (corticotroph cells) and intermediate lobe (melanotroph cells) of the pituitary to induce POMC gene expression and hormone secretion [148-150]. The pituitary POMC gene product, ACTH, is the principal mediator of basal and stress-induced glucocorticoid secretion from the adrenal cortex [147]. The glucocorticoid product inhibits its own production, via its negative feedback loop, by blocking the signals to the limbic HPA axis (brain) and pituitary (corticotrophs cells) [149-151].

Several studies suggest that neuronal POMC gene may mediate in part the inhibitory effect regulating the stress axis. This can be a possibility because PVN CRH neurons are densely innervated by POMC arcuate neurons [152]. Most importantly, a large percentage of PVN CRH neurons express melanocortin-4 receptors and respond directly to melanocortin stimulation accompanied by increased plasma ACTH, corticosterone and decreased food intake [152-155].

More interestingly, opioid peptides including β-endorphin can directly modulate the function of CRH neurons in the PVN with predominantly inhibitory actions by μ opioid receptor activation leading to alter HPA activity [156, 157]. However there is contradictory data regarding whether glucocorticoids have long-loop feedback effects on the activity of POMC neurons in arcuate neurons and in the expression of the POMC gene analogous to their clearly established inhibitory control of pituitary corticotrophs [158, 159].

The role of POMC as a dual modulator (neural POMC peptide action as an inhibitor versus the pituitary POMC peptide as enhancer) of the HPA axis is a complex mechanism to decipher. It is believed that neuronal POMC peptides may signal an inhibitory effect on CRH neurons. However present data cannot distinguish which peptide product of the POMC gene is the culprit to this effect, or whether both (α-MSH and β-endorphin) peptides are needed for this effect. Smart et al. suggested that the absence or reduction in central POMC might indirectly alter the set point for CRH neuron activity relative to circulating corticosterone levels by a polysynaptic pathway [147]. Hence, suggesting the importance of this POMC dual mechanism to mediate the HPA axis homeostasis.

The role of POMC peptide products in metabolic signaling

I. In the hypothalamus

There are several hormones that mediate the appetite center located in the hypothalamus, most of these hormones derived from other peripheral organs. For example, leptin derives from adipocytes and insulin derives from the pancreas; both signal for food intake inhibition by reducing NPY and stimulating POMC in the arcuate

neurons. However, Ghrelin derives from the upper part of the GI tract enhances appetite by stimulating NPY neurons [160, 161].

Leptin regulation of energy homoeostasis is mediated by the melanocortin system [162, 163]. In the arcuate nucleus of the hypothalamus both the POMC and AgRP expressing neurons expressed the long form of the leptin receptor. The signaling cascade is the following: when circulating leptin hormone levels are high, it binds and activates its cytokine leptin receptors on POMC neurons. This in turn, induces the JAK/STAT pathway signaling that leads to alterations in transcription of several target genes in the hypothalamus, including POMC and AgRP [115, 164, 165]. In the POMC neurons, this signal causes the release of melanocortin peptides such as α-MSH that in turn inhibit food intake [166]. Thus, when circulating leptin levels are high, leptin activates its receptors on AgRP neurons in the arcuate nucleus to inhibit the release of AgRP, except in leptin-resistant obese individuals [167-169]. In addition, ablation of leptin, its receptor, or STAT3 activation causes reduced hypothalamic expression of POMC as well as obesity and impairments in glucose metabolism [116, 169, 170]. It also has been observed that under fasting state, the levels of leptin and hypothalamic POMC gene expression are reduced [171]. Furthermore, the following conditions such as hypothalamic lesions, mutations in the tubby and nhlh2 genes, aging or insulin deficiency leads to reduction in the hypothalamic POMC gene expression [172-176] .

Insulin also influences POMC neuronal function [169]. Insulin is synthesized by pancreatic β-cells and it circulates at levels proportional to adiposity. Insulin influences POMC neurons by signaling to the arcuate POMC and AgRP neurons to regulate energy homeostasis [177] and satiety [178]. Lin et al., recently showed divergent effects of

insulin signaling in the hypothalamus on energy expenditure and glucose production. The authors demonstrated that they were able to restored insulin receptors in either POMC or AgRP neurons in mice deficient in hypothalamic insulin receptors. Moreover, the insulin signal varies in POMC versus AgRP neurons. The insulin signaling in AgRP neurons decreases hepatic glucose production, whereas insulin signaling in POMC neurons promotes hepatic glucose production and activates melanocortin-mediated increased energy expenditure [169, 179].

In addition, glucose-sensing neurons in the hypothalamus have been implicated in the control of feeding behavior, glucose homeostasis and have been associated with metabolic related diseases. Interestingly, it has been demonstrated that a subpopulation (~ 50 %) of POMC neurons in the arcuate neurons of the hypothalamus are excited by glucose, and this effect is lost with obesity linked to a high-fat diet [180, 181]. Furthermore, Zhang showed that Hypoxia-Inducible Factor (HIF) activation results in up-regulation of POMC gene. Thus, HIF loss of function in POMC neurons causes glucose desensitization to promote energy imbalance and obesity development [182]. More interesting is the effect of glucose on leptin, Ashcroft et al., explained that under low glucose levels, leptin decreases the overall excitatory tone and therefore hyperpolarizes POMC neurons and reduces their firing rate. In contrast, at 11 mM glucose, leptin increases the overall excitatory tone, and stimulates POMC neurons [183]. This high glucose effect have been shown earlier by Cowley et al., in that study, leptin stimulated POMC neurons via activation of a nonselective cation current, suggesting that leptin also interacts directly with POMC neurons [112]. Neuronal metabolism of glucose generates ATP that binds to and closes the K_{ATP} channels on POMC neurons. This leads to reduced

K+ outflow, depolarization of the POMC neuron membrane, increased electrical activity and stimulation of α-MSH release [184]. Parton et al., demonstrated that glucose was found to stimulate α-MSH secretion from hypothalamic brain slices from wild-type mice in a dose-dependent manner but not from POMC mut-Kir6.2 mouse hypothalamic slices. Although disruption of POMC-specific K_{ATP} channels in mice impaired glucose tolerance it did not change body weight [169, 181]. It remains to be elucidated the mechanisms that mediate central glucose concentrations in POMC neurons and whether this effect influences POMC peptides in peripheral organs. In humans, several mutations in the POMC gene have been associated with early onset of obesity, adrenal insufficiency, and red hair pigmentation [185, 186]. Buono's lab studied detected specific gene variants of the POMC and melanocortin receptor 4 (MC4R) genes in severely obese adults living in southern Italy. A total of 196 subjects were tested, of these participants 1.5% had mutations in POMC exon 3 (P231L, E244X, and R236G) and 2.5% had MC4R mutations (W174C, Q43X, S19fsX51, I317V, and A175T) [187]. Moreover, O'Dell study examined four (G51C, C7965T, C8246T, RsaI) polymorphisms spanning the POMC gene to test for association between POMC gene variants, serum leptin and body fat. A total of 2,758 normal females from St. Thomas' UK adult twin registry were tested, the results demonstrated that only two polymorphins (C8246T and RsaI) showed association with leptin and/or body fat deposition. The authors postulated that the association with body fat and leptin in regions flanking POMC gene represented the control of POMC expression in mediating the determination of body weight [188]. Interestingly, Keavney et al., focused on three (RsaI, C1032G, and C8246T) polymorphisms spanning the POMC gene in obese families. The total subjects were 1,428 members encompassing

248 families, the results showed that only two polymorphisms C8246T and C1032G showed significant associations with waist to hip ratio. The authors proposed that genetic variants at the POMC locus influence body fat distribution within the normal range [189].

These two studies demonstrated repeatedly the importance of (C8246T) POMC gene variant in body fat determination. Another study using 811 Hispanic subjects within the insulin resistance atherosclerosis family study (IRAS) was tested for association with multiple obesity quantitative traits. The POMC SNP C3755T showed an association with BMI, waist, visceral adipose tissue, and subcutaneous adipose tissue. However, POMC SNP G3460C, G3473A, and A7069G were found to be associated with other additional obesity measurement [190]. In conclusion, as it can be observed many forms of obesity and diabetes are associated with impairments in the production of, processing of, or sensitivity to products of the POMC gene. Thus reduction of the POMC gene may actually be a cause of the metabolic impairments. Thus we can suggest that POMC contributes genetically to the development of obesity

The role of the circadian system in POMC expressing neurons

I. The role of the circadian system

In mammals, the biological clocks are present in every cell. These clocks are considered to be the regulatory factors controlling circadian rhythms of behaviors and physiological functions. However, the master clock that coordinates these functions is located in the SCN. The SCN consists of two small bilaterally paired nuclei located in the anterior hypothalamus immediately above the optic chiasm and lateral to the third ventricle in mammals [191]. The biological clock allows the organism to anticipate and prepare for the changes in the physical environment that are associated with day and

night, thus ensuring the organism to act accordingly based upon the time of day. In addition, the biological clock ensures that internal changes take place in coordination with one another. The synchrony between external and internal environment cues is critical to the organism's survival. A lack of synchrony (such as jet-lag, shift-work, and sleep loss) within the internal environment might lead to health problems [192].

II. The molecular mechanism of the circadian clock

The molecular mechanism underlying circadian clock function is based on the interconnected transcription/translation feedback loops [192-194]. In mammals, the clock mechanism is composed of at least nine main proteins: CLOCK (circadian locomotor output kaput), BMAL1 (brain and muscle ARNT-like protein 1), PER1 (Period 1), PER2 (Period 2), PER3 (Period 3), CRY1 (cryptochrome 1), CRY2 (cryptochrome 2), REVERBα (reverse erythroblastosis virus α), and RORα (retinoid-related orphan receptor –α). Many of these proteins act as transcriptional factors, since they have PAS (Per-ARNT-Sim; involved in protein interactions) and bHLH (basic helix-loop-helix; involved in protein DNA interaction) domains [195]. These characteristics aids in the regulation of the circadian clock system.

The development of the circadian clock system starts during fetal development, however the onset of the rhythm activity is controversial. Sladek's group showed that as early as E19, all the clock genes (*Per1, Per2, Bmal1, Clock* and *Cry1*) were already expressed in the SCN; however no SCN rhythm in their expression was detected. It is at the postnatal day three (P3) that the rhythms of *Per1, Per2, Cry1,* and *Bmal1* but not *Clock* mRNA were minimally expressed in the SCN, this rhythm mature at P10 [196].

Then the same lab also found that it is at E20, that the *Per1* gene expression starts to be rhythmic whereas, the other genes remained arrhythmic till the first postnatal day [197].

Similarly, Li's group demonstrated that out of all the clock genes only *Bmal1* was prominently expressed in the fetal SCN while the other genes were expressed minimally. It was shown that the expression of these genes increased just after birth. In contrast to Sladek's work, Li's works exhibited absence of robust oscillations during early SCN development [198].

Moreover; El-Hennamy's group showed that if maternal shift occurred between the following developmental periods (E1, P0-1 or the interval between E20 and P3, it led to phase shift of the pup's SCN clock at birth [199]. Suggesting that the SCN clock is capable of significant phase shifts at fetal developmental stages when no or very faint molecular oscillations occurred.

In terms of SCN photic sensitivity, both the *Per1* and *Per2* clock genes are considered the light-sensitive components of the SCN. Mateju's worked demonstrated that expression of *Per1* was light responsive from P1 and the responses began to be gated by the circadian clock at P3 whereas expression of *Per2* was only slightly light responsive at P3, and the response was not fully gated until P5. This data reflects the importance of the postnatal period on the function of the SCN [200]. It demonstrates that the light sensitivity of the circadian clock develops gradually during postnatal ontogenesis before the circadian clock starts to control the response. Moreover, photoinduction of the clock gene *Per2* develops later than that of *Per1*.

The circadian clock system requires two feedback loops to maintain the 24 hr. cycle. Therefore there are two loops that mediate the cycle: the positive and negative

feedback loop. The positive elements of the central oscillatory loop are CLOCK (or homolog NPAS2 – which is expressed in the forebrain and vasculature) and BMAL1. The CLOCK/BMAL1 heterodimer binds to E-box elements in the promoters of target genes and drives rhythmic transcription of three *Period* (*Per1, Per2, and Per3*), two *Cryptochrome* (*Cry1 and Cry2*) genes [201], *Reverbα, Rorα,* and many output genes that are controlled by the clock (CCGs: clock-controlled genes) [202]. The heterodimer CLOCK/BMAL1 also stimulates Bmal1 transcription. Conversely, when PER and CRY proteins are translated, they form PER/CRY complexes that are translocated to the nucleus where they inhibit CLOCK/BMAL1-mediated transcription, forming a negative autoregulatory loop. As a result, transcription of the *Per* and *Cry* genes is reduced, which results in decrease in the abundance of the corresponding proteins. This leads to release of the repression of CLOCK/BMAL1 and to initiation of a new, 24-hour transcriptional cycle [193, 194, 201, 203].

Studies have proposed that both sirtuin 1 and NAD+ are components of a new negative feedback loop in the circadian clock system. Sirtuin 1 gene expression is dependent on NAD+ levels. Therefore Nampt (nicotinamide phosphorobosyltransferase), an enzyme that produces nicotinamide mononucleotide from nicotinamide and 5'-phosphoribosyl 1-pyrophosphatase - a rate limiting step in NAD+ synthesis; is the mediator for SIRT1 because NAMPT induces the production of NAD+. Interestingly, cellular NAD+ oscillation is in phase with SIRT1 deacetylation activity however NAD+ phase is opposite to that of acetylation of histone H3 and BMAL1 [204-206].
SIRT1 and NAMPT may mediate the circadian clock system in two ways: 1. By inducing deacetylation of both BMAL and PER2 causing disruption of the CLOCK: BMAL1

heterodimer that drives the transcription of Per2 gene expression. 2. SIRT1 can also bind to the E- box of the Per2 promoter that inhibits the Per2 gene expression levels. 3. PER2 becomes acetylated by CLOCK but deacetylation occurs by SIRT1 interaction this enhance the rate of PER2 degradation. 4. Interestingly, in nonsynchronized Sirt1 KO MEF (mouse embryonic fibroblast), levels of endogenous *Bmal 1* mRNA were reduced to 40%, BMAL and CLOCK protein levels were downregulated, *Per 2* mRNA level were decreased but the PER2 protein accumulation was higher in these cells [207-210]. This demonstrates that SIRT1 may be a point through which changes in cellular energy metabolism impact the functioning of the clock.

III. The development of the arcuate nucleus projection pathways

Neurons in the arcuate nucleus are generated from the ventral part of the periventricular neuroepithelium of the hypothalamus between embryonic day 12 (E12) and E17 [211], with POMC and NPY neurons appearing as early as E12 [212] and E15 [213] respectively. Even though, these neurons are developed during the embryonic developmental phase, Bouret's group revealed that its projection pathways are not present at birth, however they develop entirely postnatally. Moreover, these projections are not mature until the third week of life when pups begin to leave the microenvironment of the nest and search for solid food [214]. This finding suggest that the neonatal period is a critical stage of development for the arcuate neurons (a site known for metabolic signaling), during which animals need to maximize caloric intake and maintain appropriate metabolic responses to ensure growth and survival. This is interesting because the postnatal period is also important for the circadian clock signaling to mature its signaling transmission, sets its rhythmicity pattern, and respond to its environment

cues. Therefore any disruption to the hypothalamus both the SCN and arcuate nucleus may affect the signaling patterns of these nuclei that may lead to physiological abnormalities.

When does homeostatic regulation begin? Studies indicated that leptin does not exert its anorectic effect until the third week of life. Intracerebroventricular, or peripheral, injection of leptin to 9 days old pups failed to influence milk intake or body weight, whereas by 28 day of age, administration of leptin inhibited food intake [215, 216]. In addition, neonatal leptin-deficient mice are not overweight, unlike adults [215], but chronic treatment of neonates with exogenous leptin regulates expression of arcuate neuropeptides implicated in energy homeostasis such as POMC and NPY[216]. Although, these neurons seemed to lack the leptin signal transmission in neonatal animals, one can suggest that any disruption (such as chronic neonatal ethanol exposure) to the process of neuronal maturity and its projection during this critical phase can lead to altered neuronal signaling by the arcuate neurons that may be observed at the adulthood phase as suggested by previous studies.

It seems that the arcuate neurons does not only innervate the hypothalamic dorsomedial nucleus (DMH), paraventricular nucleus (PVH), lateral hypothalamic area (LHA) ([214], but also the site that maintains the central clock. Neuroanatomical tracing studies in the ARC and SCN showed that the SCN receives direct input from the arcuate neurons and median eminence and vice versa. This means that input from the SCN to the ARC may time the activity of the ARC [217]. This is an implication that the ARC may relay peripheral hormonal information to the SCN and mediate an effect to the circadian activity of the SCN. Thus metabolic cues could indeed influence circadian activity and

vice versa leading to a serious risk of an unbalanced energy state and possibly obesity, diabetes or others metabolic diseases [218, 219]. Furthermore, gene expressions of circadian clock genes were found in the SCN and MBH. In addition, the expression patterns of clock genes in the MBH were stable under various light conditions [220]. The next question will be whether POMC expressing neurons are driven in the circadian manner?

IV. POMC system function and the circadian clock mechanism

The aforementioned data suggests that the arcuate nucleus of the hypothalamus receive signals from the SCN, the central clock oscillator. Indeed, POMC neurons have been shown to have a rhythmicity pattern. The daily activity of MBH POMC neurons fluctuates throughout a 12 h light: 12 h dark (12L: 12D) cycle. In fact, the expression of POMC mRNA undergoes day and night oscillations in both male [221] and female [222] rats. In male rats, lowest levels of MBH POMC mRNA expression are detected at dark onset and peak levels are detected at dark offset. At the peptide level, the MBH content of β-endorphin also displays daily fluctuations in male rat [223], female rat [224], and male Syrian hamster [225]. In the male rat, this content is high during light phase, low at early night, high again at late night, following a pattern that correlates with the immuno - detectability of β-endorphin containing perikarya. More interestingly is the findings by Tramu et al., which showed that Fos –expressing, POMC-containing neurons mediate resetting/synchronizing actions of both clock and LD cues upon neuroendocrine and behavioral rhythms. The authors suggest that this synchronization may be the way the POMC expressing neurons may mediate the hormone release in order to accommodate

for metabolic timing and stress related behavior [226]. If that is the case, disruption in the POMC circadian expression may lead to alteration to mediating metabolic signaling.

Interestingly, Chalmers et al., using serum shock synchronization showed that POMC gene expression was rhythmic in Movas-1 (vascular smooth muscle) cells. Furthermore, the POMC gene expression profile mimicked the pattern of BMAL1 in these cells. The authors predict that the clock system, which is mediated by several transcription factors, may directly control POMC expression in these cells [227]. If so, the questions is which of these transcription regulators may be responsible for this regulation in this cells, whether this regulations is mimicked in other POMC expressing cells too, and if that is the case does these transcription factor also mediate metabolic related genes that may be expressed where the POMC gene is expressed?

Genes involve in metabolic related function in POMC-expressing neurons.

It is known that through efferent projections from both NPY/AgRP (orexigenic) and POMC/CART (anorexigenic) neurons mediate the metabolic signaling. In the case for POMC expressing neurons, which inhibits food appetite, it is believe that metabolic genes expressed in POMC expressing neurons aid in maintaining this anorexigenic state. For that reason, we focused on these four genes STAT3, ASB4, SIRT1, and PGC1α that have been previously shown to play a role in cellular metabolism.

I. STAT3

In the hypothalamus, several studies have elicited the role of STAT3 in metabolic function. For example, leptin administration caused rapid phosphorylation of Stat3 [228]. Consequently, a neural-specific deletion of Stat3 using Cre-recombinase transgene driven by Nestin promoter caused a severe obesity phenotype and reduced the expression of

POMC [116]. Furthermore, removal of activated Stat3 from POMC neurons resulted in reduced levels of POMC mRNA. These findings suggest that STAT3 is essential for maintaining metabolic signaling and modulating POMC expression [170].

II. ASB4

The ASB4 gene is a member of the ankyrin repeat and SOCS box containing protein family (Asb-1 to Asb-18). Proteins of the ASB family are a component of E3 Ub ligase and responsible for recognizing specific cellular proteins for ubiquitination [229]. Other than human, ASB4 has also been found in a wide range of organisms including zebrafish, mouse, rat, cattle, chicken, monkey, chimpanzee and dog. ASB4 is highly conserved between human and mouse and these two species share a 93% amino acid homology with each other [230].

ASB4 is expressed in the hypothalamus, predominantly in areas specific to metabolic function [231]. All Asb proteins have two functional domains, a C-terminal SOCS box region of approximately 40 amino acids and an upstream ankyrin repeat region [232]. Unlike the other Asb proteins, Asb-4 only contains nine ankyrin repeats N-terminal to its SOCS box [230].

Asb-4 contribution to metabolic signaling were revealed by Li's group, this group demonstrated that in the fed state, Asb-4 mRNA is expressed by 95.6% of POMC neurons and 46.4% of neuropeptide Y (NPY) neurons. In contrast, in the fasting state, the percentage of POMC neurons expressing Asb-4 mRNA drops to 73.2% ($P < 0.001$). Moreover, the density of Asb-4 mRNA per fasted POMC neurons is markedly decreased [231, 233]. Conversely, expression of Asb-4 mRNA by NPY neurons in the fasted state is modestly increased to 52.7% ($P < 0.05$) [233]. Based on ASB4 differential expression,

neuroanatomical distribution and co-localization, one can predict that Asb-4 plays a major role in energy homeostasis, mainly in the hypothalamus [233].

Interestingly, in recent study Li's group demonstrated that Asb-4 co-localizes and interacts with IRS4 in hypothalamic neurons [234]. IRS (insulin receptor substrate) proteins play a central role in signal transduction by insulin, IGF-1, and a growing number of cytokines [235]. These proteins serve as multi-adaptor proteins and are phosphorylated on multiple tyrosine residues to mediate SH2-protein recruitment and downstream signaling [236]. Particularly, IRS4 is highly expressed in the hypothalamus [237, 238]. Interestingly, Fantin's group demonstrated that IRS4 null mice exhibited slightly lower blood glucose concentration than the wild type in both fasted and fed states, but the plasma insulin concentrations of the IRS4 null mice in the fasted and fed states were normal. IRS4 null mice also showed a slightly impaired response in the oral glucose tolerance test. Thus, mice lacking IRS4 exhibited mild defects in growth, reproduction, and glucose homeostasis [239].

Li's group showed the following: 1. Asb-4 interacts with IRS4 in cell lines. 2. It mediates the degradation of IRS4. 3. Asb-4 decreases insulin signaling [234]. Thus, inhibition of insulin signaling by Asb-4 interaction with IRS4 may partially explain the hyperphagia in the POMC-Asb-4 transgenic mice since insulin can inhibit food intake in the brain. Moreover, Asb-4 action to direct degradation of IRS4 may be a mechanism by which NPY and POMC neurons modulate the sensitivity to circulating levels of insulin and glucose.

III. Sirtuin 1

Sirtuin1 (SIRT1) is a nicotinamide adenosine dinucleotide-dependent deacytalase that orchestrates key metabolic adaptations to nutrient deprivation in peripheral tissues [240]. Recent studies suggest that SIRT1 is localized in the CNS, mainly in the arcuate nucleus of the hypothalamus. By using immuhistochemistry techniques investigators have shown that SIRT1 colocalizes with POMC neurons (done by using Pomc-GFP mice) [241]. Its function in the brain appears to regulate food intake and feeding behavior. Numerous experimental and clinical findings suggest that hypothalamic dysfunction might be one of the underlying causes of abnormal glucose and lipid metabolism that occurs in type 2 diabetes and diet-induced obesity [133, 242].

Furthermore, deletion of Sirt1 in POMC expressing neurons results in weight gain and reduced energy expenditure by reducing sympathetic nerve activity and BAT (brown adipose tissue)-like remodeling of perigonadal WAT (white adipose tissue) under high fat diet [243]. These results suggest that SIRT1 in POMC-expressing neurons is required for normal autonomic adaptation to direct induced obesity and possibly insulin resistance [244].

In addition, SIRT1 can also regulate the activity of transcription factors such as PPARγ, p53, and the FOXO family of transcription factors, all of which are key regulators of metabolism in a variety of tissues [245]. Cakir's group showed that a fasted state yields an increase in in hypothalamic Sirt1 expression and decreases forkhead transcription factor (FoxO1) acetylation suggesting that Sirt1 regulates the central melanocortin system in a FoxO1 dependent manner [246, 247]. Interestingly, *In vivo* studies using Sirt1 siRNA technique in the arcuate nucleus showed an increase in the proACTH intermediate peptide. Similarly, *in vitro* studies using AtT20 cell lines, which

were exposed to drug treatment in order to induce SIRT1 expression showed a decrease in the POMC protein level. These findings demonstrate that Sirt1 plays an inhibitory role on POMC gene regulation. Lastly, SIRT1can also directly interact and deacetylate PGC1α [248]. Studies have shown that in response to fasting signals, SIRT1 induces gluconeogenic genes and hepatic glucose output, but represses glycolytic genes through deacetylation of PGC1α [249]. The interest of these energy sensing proteins is derived from the evidence that they are regulated by several factors including food intake and are precisely up-regulated by food restriction.

IV. PGC1α

Peroxisome proliferator-activated receptor-γ-coactivator-1-α (PPARGC1A, aka PGC1α) is part of a small family of transcriptional coactivators that includes the close homologs PGC1β and PGC1-related coactivator [250]. This gene is localized on chromosome 4p15.1-2, a regions that has been associated with basal insulin levels, abdominal subcutaneous fat, and obesity[248] . The function of coactivators is to provide specificity by targeting tissue and specific cell type subsets of nuclear receptors to specific promoters. The regulation of these coactivators may occur through different mechanisms including translocation between cytoplasm and nucleus, post-translational modifications such as phosphorylation and acetylation, and regulated proteolysis [251]. This family of coactivators contains the following characteristics: (1) the N- and C-terminal regions are similar in all these co-activators. These areas contain most of the effector domains. (2) It contains leucine-rich motifs (LXXLL domains) that serve to mediate the interaction with hormone receptor binding domains. (3) All coactivators of

this family have an RNA-binding motif. (4) Specifically, PGC1α contains a serine/arginine-rich domain that regulates RNA processing [251].

PGC1α is mainly expressed in tissues with high energy oxidative capacity like heart, skeletal muscle, liver, brown adipose tissue and brain. Studies have revealed that conditions that require energy such as cold, fasting and exercise enhanced its expression [248]. PGC1α was first identified as a protein of 798 amino acids that interacts with the nuclear receptor PPARγ (peroxisome proliferator-activated receptor- gamma), the master regulator of adipocyte differentiation. Interestingly, the LXXLL motif on PGC1α is absolutely required for the ligand-dependent interaction with ER, PPARα, RXRα, glucocorticoid receptor, and probably other nuclear hormone receptors.

Furthermore, studies have shown that several environmental conditions can have a variable effect on PGC1α in different tissues. For example, during a fasting state PGC1α is increased in the following tissues hepatic, breast muscle, subcutaneous fat, cerebrum, but decreased in the pituitary and anterior hypothalamus (where the SCN is localized)[250, 252].

In addition, PGC1α expression is induced under both model type 1 (insulin deficient) and type 2 diabetes (high insulin levels but profound insulin resistance), and in ob/ob mice. Moreover, studies have revealed that PGC1α activates the gluconeogenesis process by stimulating the mRNA expression of all three key genes in gluconeogenesis (PEPCK, fructose 1,6-bisphosphatase, and glucose-6-phosphatase) [250].

Animal studies have revealed that in mice having liver-specific mutations in the insulin receptor, the LIRKO, the hepatic PGC1α gene expression were elevated. Thus, suggesting that insulin represses the hepatic expression of PGC1α [250]. How insulin acts

to suppress PGC1α expression and whether this action of insulin is direct or via the counter-regulatory hormones controlled by insulin such as glucagon and the glucocorticoids is still unknown.

Clinical studies have shown that individuals with type-2 diabetes exhibit decrease expression of PGC1α in skeletal muscle; this inhibition may be due to reduced oxidative gene expression in this disease. Similarly, elderly adults show significantly lower PGC1α expression in skeletal muscle compared with young adults. Several SNPs (G482S) in PGC1α have been investigated for their relationship with diabetes. Interestingly, Lai's group examined the Boston Puerto Rican Health study and correlated the existence that increase physical activity reduced DNA damage [253]. Thus the author's postulated that exercise may increase the PGC1α expression leading to a decline in the incidence of cardiovascular disease in individuals with type 2 diabetes.

Liu's group rendered characterization between PGC1α and the circadian clock. The group revealed that PGC1α stimulates the expression of *Bmal1* through coactivating the ROR family of orphan nuclear receptors and is essential for normal circadian rhythms. The results showed that PGC1α activates *Bmal1* transcription by altering the local chromatin environment from a repressive to an active state. More interesting is the PGC1α null mice phenotype, the mice are resistant to diet induced obesity and are more insulin sensitive. This may be due to the fact that these mice are hyperactive and have a higher metabolic rate in the absence of increase food intake [254]. Since feeding and locomotor activity are regulated by circadian clocks, it is possible that rhythmic PGC1α expression is controlled by components of the clock oscillator.

Effects of ethanol on the POMC system, metabolic related signaling and circadian system

I. Effects of ethanol on the POMC system:

Several reports identified a link between energy regulatory genes and alcohol exposure. Nyomba et al., 2006 demonstrated that prenatal ethanol exposure exaggerates the expression of gluconeogenic genes in adult rat offspring [38]. The prenatally alcohol exposed rats also developed insulin resistance in both juvenile and adult periods, explaining the increase in gluconeogenesis. In addition, it has been shown that β-endorphin producing cells in the hypothalamus are critically involved in regulation of food intake and metabolism [255], and chronic alcohol administration reduces β-endorphin secretion and POMC gene expression [256] [50]. These data provide strong evidence that prenatal alcohol exposure may alter the metabolic signaling at least partly by altering the function of POMC-producing cells in the hypothalamus.

II. The POMC biological peptide: ACTH

Both clinical and biochemical evidence suggests that alcohol exposure may incur endocrine dysfunction [257, 258]. For example, animal studies revealed that chronic exposure during pregnancy elevated the basal level of corticosterone in fetus and caused a hyper-responsiveness of HPA axis in offspring [9, 259, 260]. In humans, high adult plasma cortisol levels are found in individuals with low birth weight, and may contribute to the increased cardiovascular risk observed in these individuals [261]. This is interesting because FAS patients have these characteristics too.

Studies have shown that prenatal alcohol exposure leads to hyperresponsive to stressors and drugs during the adulthood period by exhibiting increased corticosterone, increase CRH mRNA levels, increase anterior pituitary POMC mRNA levels, increase

ACTH hormone, and CRH (corticotropin releasing hormone) responses to stressors such as restraint, foot shock, LPS or ethanol challenge [27, 52]. This response may be due to alterations in the POMC signaling pathway. Studies have shown that gestational chronic ethanol treatment reduces the number of β-Endorphin neurons during the developmental period.

β-Endorphin expressing neurons in the arcuate nucleus are known to innervate the paraventricular neurons in order to inhibit the release of CRH. If this signal is damage, the inhibitory influence of β-Endorphin on CRH release is loss, leading to uncontrolled CRH release that may result in induction of pituitary corticotrophs cells to synthesize and release ACTH, leading to a hyper-responsive phenotype. FAS patients and ethanol animal models exhibit this phenotype.

III. The POMC biological peptide: β-endorphin

POMC – expressing neurons are located primarily in the arcuate nucleus of the mediobasal hypothalamus. The opioid peptide β-endorphin is one of the primary POMC-derived peptides in the hypothalamus and has been implicated in regulation of a variety of brain functions related to drug abuse, including psychomotor stimulation, positive reinforcement, and mood disorders [255, 262-264]. This peptide also plays a role in prenatal ethanol induced growth retardation, behavioral abnormalities, central nervous system damage, undernutrition, hypoxia, defects in learning and memory, and neuroendocrine abnormalities [265-268].

The functional changes observed in the β-endorphin neurons following ethanol treatments paralleled many behavioral changes observed following alcohol abuse in humans [256, 269-275]. *In vitro* studies demonstrate that under acute conditions, ethanol

administration increases the release of immunoreactive β-endorphin in hypothalamic neuronal cultures. However, upon chronic ethanol administration this effect is decreased [272, 276]. The desensitization effect of chronic ethanol raises the possibility that the hypothalamic endorphinergic system may be involved in the development of tolerance and physical dependence on ethanol [256, 273].

Studies by Chen et al. associated the opioid system, the circadian system and alcohol consumption. Their data demonstrated that both chronic and prenatal ethanol exposure alters the circadian rhythm of (POMC) mRNA expression in the arcuate nucleus of the hypothalamus. Similarly, it alters the circadian expression of the clock governing genes (e.g., rPeriod1; rPeriod2 and rPer3 mRNA) in the arcuate nucleus and SCN [50, 51]. Moreover, chronic ethanol treatment disrupts gene expression profile of Per genes in the brain. The gene expression profile of rPer1 in the arcuate nucleus demonstrates a robust circadian rhythm in control rats, whereas its circadian rhythm is lost in ethanol treated groups. Similarly the circadian rhythm pattern of rPer2 mRNA expression is significantly different in ethanol treated groups; it reduces the circadian amplitude of rPer2 mRNA expression in the SCN compared to control. The rPer3 mRNA expression exhibits a robust circadian rhythm in control groups, but this observation is not seen in ethanol-fed group [50]. This means that prenatal ethanol exposure disrupts the central clock (SCN) and other clocks (arcuate nucleus) [51]. As a result, this data implicates the involvement of the circadian and opioid system in ethanol action.

Furthermore, human and animal studies suggest that the endogenous opiod peptide, β-endorphin, is linked to alcohol abuse [270]. One of the ways that alcohol can

influence brain function is by increasing or decreasing the release of a number of neurotransmitters in distinct brain regions, among these are the β-endorphin peptides.

IV. The POMC biological peptide: α-MSH

The melanocortin system (MC) components are products of POMC. Recent pharmacologic and genetic evidence suggests that MC receptor signaling modulates neurobiologic responses to ethanol and ethanol intake [277, 278]. Studies have revealed that chronic ethanol exposure reduces POMC biosynthesis in the pituitary gland and in the CNS. Therefore, under chronic ethanol exposure reduction of α-MSH is observed in the pituitary gland, in the arcuate nucleus of the hypothalamus and in the substantia nigra [277]. Navarro's group observed that there were no differences in body weights or caloric intake between the control diet and ethanol diet groups, this suggests that reductions of α-MSH expression in the ethanol group is due to ethanol exposure rather than altered energy balance [278]. Interestingly, recent work from Navarro 's group revealed that ethanol targets POMC at a site specific by reducing the expression of prohormone 1/3 in the arcuate nucleus whereas prohormone 2 and central β-endorphin showed no differences [279]. These findings suggest that ethanol may target POMC gene at the posttranslational modification level.

V. Effects of ethanol on metabolic related genes:

Ethanol exposure alters gene expression levels of a variety of genes; its effect on gene expression can be centralized or global. Several studies have shown the following genes STAT3, ASB4, SIRT1, and PGC1α are targets of ethanol outside the CNS.

A. STAT3

In the hypothalamus, STAT3 serves as a component of the leptin-signaling pathway. The leptin receptors become active upon stimulation of the signal transducers and activators of transcription (STAT) second messenger system. Under ethanol exposure, the following results were observed: 1. In the hypothalamus, the long form of leptin receptor and STAT3 protein expression was reduced [280]. 2. Inhibited the leptin-induced STAT3 phosphorylation [281]. 3. Suppressed leptin gene expression in white adipose tissue and reduced the plasma leptin concentration at all times measured and it perturbed genes involved in fatty acid β-oxidation[282] . This suggests that ethanol exposure affects metabolism by altering the leptin system that regulates energy balance.

B. ASB4

Alcohol intake has been definitely recognized as a cause of chronic liver diseases, including hepatocellular carcinoma (HCC)[283]. Alcohol could be involved in the development of HCC through both direct (genotoxic) and indirect mechanisms. An indirect mechanism includes the development of cirrhosis, which is probably the most common pathway to liver carcinogenesis in developed countries [284]. Intriguingly, studies have demonstrated that ASB4 may serve as a diagnostic marker for HCC due to its high expression in tumor tissues of HCC patients. ASB4 expression in HCC was significantly higher when compared with their non-tumor counterparts [229], this implies the contribution of this molecule to the cancerous phenotype of HCC. In addition, ASB4 was reported to inhibit the JNK pathway through its interaction with the GPS1 protein [231]. Since there are evidences supporting the tumor suppressive role of JNK pathway when activated [285], ASB4 may contribute to cancer development through its inhibition on the JNK pathway.

C. SIRT1 and PGC1α

The following studies demonstrated the role of hepatic SIRT1 and PGC1α in response to ethanol [286, 287]. Lieber's group showed that chronic ethanol exposure reduced both gene and protein expression levels of hepatic SIRT and PGC1α, two key proteins involved in energy regulation and mitochondrial homeostasis, FoxO1 gene expression levels were increased whereas the phosphorylated form of FoxO1 and Akt were reduced, and PGC1α was hyperacetylated in compare to control group [288]. This is interesting because the pathway for the oxidation of ethanol is led by alcohol dehydrogenase (ADH). ADH results in the production of acetaldehyde and reduction of nicotinamide adenine dinucleotide (NAD+) to its reduce form (NADH). SIRT1 is an NAD-dependent deacetylase, its reduction can exacerbate even further the alcohol-induced changes in the lactate/pyruvate ratio which alters the NAD+/NADH ratio with a decrease in NAD+ leading to mitochondrial dysfunction. A high concentration of NADH hinders the hepatocyte ability to maintain redox homeostasis and mitochondria function thus increasing the incidence of metabolic related disorders.

In addition, You's group revealed that reduction of SIRT1 by ethanol exposure induces the acetylation of nuclear SREBP-1, a protein that increases the synthesis of hepatic lipid. Interestingly, a combination between a high saturated fat diet and ethanol exposure resulted in increased SIRT1 protein and reduction in SREBP-1. Therefore, high fat diet may suppress the expression of genes encoding lipogenic enzymes resulting in decrease in synthesis of hepatic fatty acids.

The latest SIRT1studies revealed a new target of ethanol action, microRNA-217 (miR-217). Yin's group showed that under chronic ethanol exposure miR-217 levels are increased, which in turn leads to fat accumulation. Further analysis revealed that both SIRT1 and SIRT1-regulated genes encoding lipogenic or fatty acid oxidation enzymes were also impaired.

In the case for PGC1α, Frances's group demonstrated that PGC1α G482S polymorphism is associated with alcohol consumption in the Mediterranean population. Their study also revealed that the incidence of G alleles increased in the groups with higher alcohol intake.

All of these findings revealed the importance of these metabolic genes in ethanol action either at the tissue and cellular level.

VI. Effects of ethanol on circadian mechanisms:

Ethanol alters the homeostasis of various physiological processes, For example, alcohol consumption alters the circadian control of core body temperatures in humans and laboratory animals [289]. Alcohol deregulates the thermoregulatory system by altering the set point for body temperature, the function of the SCN does not function directly in maintaining normal body function, rather it works at a higher level to control the overall generation of the circadian rhythm, which constantly underlies these important body functions [289, 290]. Furthermore, in high-alcohol-preferring and low-alcohol-preferring mice differential circadian activity rhythms have been observed [291].

Studies have shown that adults and adolescents exposed to alcohol during their prenatal period manifest sleep-wake disturbances and stress hyper-responsiveness [26, 292]. Furthermore studies show that Jet-lag alteration and altered sleep pattern increase

the incidence of drinking behavior by causing phase shifts in body's normal rhythms [293-297]. In addition, *Period 2* (*Per2)* mutant mice drinks more alcohol than control group demonstrating that disruption in the circadian clock system may lead to increase in alcohol consumption [298].

The Per homologs (*Per1, Per2, and Per3*) were found by searching computer databases of expressed sequence tags (ESTs), and cloning and characterizing DNA surrounding sequences with homology to either *Per* genes. Animal experiments show that at various lighting conditions the mRNAs encoding all three *Per* homologs undergo 24-h oscillations in the SCN over the course of a complete LD cycle and that these oscillations continue in constant darkness. In addition, expression of the *Per* genes peaks during the day and reach a nadir during the night [191]. Mouse mutations targeted to the mPer2 gene, show altered circadian functions, suggesting a principal role of this gene in the central circadian machinery, and arguing against the notion of this gene being driven by the clock [299]. Genetic expression analysis revealed that *Per2* and *BMAL1* mRNA levels are always antiphasic in the SCN and peripheral tissues [191]. Oishi et al., 2000 and Shearman et al., 2000, suggested that *BMAL1* activates *mPer2* transcription, and that mPER2 in turn activates *Bmal1* transcription and represses CLOCK-BMAL1-mediated transcription [299, 300]. Clinical studies have provided evidence that individuals with a specific set of polymorphisms in the *Per2* gene have a greater incidence of alcohol abuse; similarly *Per2* knockout mice demonstrated high preference for alcohol [298]. These findings suggest that *Per2* plays an important role in alcohol consumption. How the *Per2* gene may regulates other genes (ex: POMC) that may be involved in addiction and metabolism remains to be unknown.

HYPOTHESIS

Taken into consideration that POMC peptides control metabolic function and that fetal alcohol affects POMC peptide expression. We hypothesize that fetal alcohol exposure alters metabolic signaling in POMC neurons.

To test this hypothesis we proposed the following:

Objective 1: Determining the role of *Period 2* gene in mediation of postnatal ethanol effect on the POMC and metabolic genes in the hypothalamus at various developmental phases.

A. To determine whether postnatal ethanol induces alterations in POMC neuronal functions

B. To evaluate the effect of Period 2 gene deletion on postnatal alcohol programming of POMC and metabolic gene expression in the hypothalamus

Objective 2: Whether Period 2 gene alters the postnatal ethanol effect on the circadian expression of metabolic genes in POMC neurons

A. Effect of Period 2 gene deletion on postnatal alcohol programming of circadian expression of metabolic genes in *Pomc*-expressing neurons

CHAPTER 2

To determine whether postnatal ethanol induces alterations in *POMC* neuronal functions

INTRODUCTION

There are clinical reports uncovering a circadian rhythmicity between alcohol intoxication and overdose [301]. There also appears to be a relationship between alcohol drinking behavior and altered body circadian functions. In line with these views, alcohol intake has been shown to alter circadian blood pressure [302, 303], core body temperature [304] and hormone release in humans [305], circadian phase shifting and free running period in mice [297], circadian phase resetting and nocturnal activity patters in the hamster [306], and rhythmicity in hypothalamic proopiomelanocortin (POMC) neurons in rats [51, 307]. Maternal alcohol drinking profoundly and permanently affects circadian function in the offspring. It is known that prenatal ethanol exposure alters core body temperature and corticosterone rhythms [29], NK cell functions rhythms [55], phase shifting ability [46], and rhythmic pituitary-adrenal function in rats [308] as well as circadian blood pressure and heart rate of adolescent human [309].

Why is the body's circadian system so vulnerable to alcohol toxicity? One logical explanation for the circadian system vulnerability may point to a possible alcohol specific target for the gene(s) that regulate the circadian functions. The circadian clock-work involves the interaction of the so-called clock genes, such as the *Period* (*Per*1, *Per*2, *Per*3), *Clock*, *Bmal*1, *Cryptochrome* (*Cry*1, *Cry*2) genes, into two tightly coupled transcriptional and translational feedback loops, thereby self-sustaining a circadian period of activity of the cell and modulating the expression of clock-controlled genes [200]. Of these genes, *Per*2 gene appears to be a putative target for alcohol. Support for this view

comes from evidence showing that chronic alcohol exposure alters the circadian expression of Period genes in the hypothalamus of adult rats [50]. Similarly, prenatal alcohol feeding alters circadian expression of Period genes in the hypothalamus and peripheral tissues [51, 55, 310-312]. Additionally, *Per*2 mice mutants (*Per*2^{Brdm1}) display enhanced alcohol consumption and preference [298], whereas *Per*1^{Brdm1} mutant mice did not show such an enhancement in alcohol drinking behavior [313]. Furthermore, alcoholics with a specific set of polymorphisms in the *Per2* gene consume less alcohol than alcoholics without the polymorphisms [293, 298]. Hence, *Per*2 appears to be a targeted gene where alcohol may act on to alter circadian functions. In order to more closely elucidate the role of the circadian clock in ethanol's effects on the brain, we examined the effect of the *Per*2 mutation on hypothalamic β-endorphin neuronal sensitivity to ethanol.

MATERIALS AND METHODS

Animal use

Per2 mutant (*mPer2*^{Brdml}) and wild type male and female mice of the same genetic background (C57BL/6J) were obtained from The Jackson Laboratory (Bar Harbor, ME). *mPer2*^{Brdml} mice carry a mutant *mPer2* gene with a deletion in the PAS dimerization domain, which is critical for the interaction with other clock proteins [314], thus rendering a non-functional PER2 protein. Although the *Per*2^{Brdml} mutant mice has an albino phenotype, because it was engineered with a tyrosinase gene deficiency that affects the ability to make melanin, the *Per*2^{Brdml} mutant mice has the same C57BL/6 genetic background with only a difference of the aforementioned gene deficiency. It has been shown that albino mice strains (129/J, RF/J, SWR/J, AKR/J, A/J, and BALB/cByJ)

that have a tyrosinase gene deficiency have no significant relationship between albinism and mean τ_{DD}, the endogenous (free-running) period of the circadian pacemaker measured in constant environmental darkness [315]. This suggests that the albino phenotype trait will have no implications to any circadian related studies. Similarly, gradual ethanol exposure has been shown to produce an increase in alcohol preference in both C57BL/6 mice with normal tyrosinase activity and in BALB/cByJ mice with tyrosinase deficiency [316, 317]. Furthermore, these two strains (C57BL/6 and BALB/cByJ) showed no differences in their hypothermic response or the brain level of cGMP to the same ethanol dose [309]. These data support that the tyrosinase deficiency will have very little consequences in alcohol-response studies. Mice were maintained under constant environmental conditions on a 12 h light/12 h dark cycle (lighting period from 7:00 a.m. to 7:00 p.m.) and ad libitum food and water. The *mPer2*^{Brdml} mutant mice were routinely genotyped to verify the *Per2* gene mutation. The primers used for detecting the Per2 gene were the following: forward1-cttgggtggagaggctattc, forward 2-cattgggaggcacaagtcag, reverse1- aggtgagatgacaggagatc, and reverse 2-gagctgcgaacacatcctca. Male and female *mPer2*^{Brdml} or male and female C57BL/6J were bred to produce neonates for MBH cell cultures or in vivo studies.

In vivo studies

In the in vivo study, postnatal day-2 (PD2) old C57BL/6 pups and Per2 ^{Brdml} mutant pups (both sexes) were fed by intubation with milk formula containing alcohol (alcohol-fed) or an isocaloric volume of maltose dextrin (pair-fed) as originally described by Goodlet [318] and modified by Sarkar [52], or pups were undisturbed (*ad libitum*-fed*)*. The alcohol–fed groups were given a milk formula containing ethanol

(11.34%; vol/vol; 0.1-0.2 ml/animal; during a period of 1 minute), yielding a total daily ethanol dose of 2.5g/kg. The feeding was conducted at 1000 and 1200 h daily for either 1 day at PD7 for acute ethanol administration or for 5 days between PD2-7 for chronic ethanol administration. Tissue samples were collected from all groups at 1300 h on PD7. The amount fed to the animals equaled 33% of the mean body weight (milliliters per gram). After feeding, the pups were immediately returned to the litter. One hour after the last feeding, six of each *ad libitum*-fed (AD), pair-fed (PF) or alcohol-fed (AF) neonates from six separate liters were sacrificed, their brains dissected, mediobasal hypothalami (MBH) were obtained and immediately frozen for hormone determination. Animal care and treatment were performed in accordance with institutional guidelines and complied with the National Institutes of Health policy. The Rutgers Animal Care and Facilities Committee approved this animal protocol.

Mediobasal hypothalamic cell cultures

The method for mouse MBH cell cultures was adopted from the previously published methods by our laboratory [256] for rat MBH cells. In brief, C57BL/6 and *Per*2 mutant neonates (both sexes) were sacrificed at day 1, and the fetuses were removed by aseptic surgical procedure. Brains from the fetuses were immediately removed and the MBHs were separated and placed in ice-cold Hanks' Balanced Salt Solution (HBSS) containing antibiotic solution (100 U/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml amphotericin B), 0.1% bovine serum albumin, and 200 µM ascorbic acid (all from Sigma-Aldrich, St. Louis, MO). The block of the MBH extended approximately 1 mm rostral to the optic chiasma and just caudal to the mammillary bodies, laterally to the hypothalamic sulci, and dorsally to ~2 mm deep and did not contain SCN. This part of the

hypothalamus is known to contain neuroendocrine neurons, including ß-endorphins, dopamine, thyrotropin-releasing hormones, and growth hormone-releasing hormones as well as glial cells [319]. We have previously characterized the similar MBH culture system in regard to β-endorphin neuronal responses to ethanol. In MBH cultures, ethanol acutely stimulates β-endorphin secretion and POMC mRNA levels while chronically inhibiting β-endorphin secretion and POMC mRNA levels[256, 320]. The β-endorphin neuronal responses to acute and chonic ethanol treatments in MBH cell cultures are similar to those seen in hypothalamic tissues of adult rodent brains [321-323]. Also in MBH cell cultures, like in the adult hypothalamus, ethanol action on β-endorphin neurons is mediated by catalase-activated production of acetaldehyde [324, 325]. These data support that the MBH cell culture model is useful in determining cellular mechanisms of ethanol actions on β-endorphin neurons.

MBH cells were then sedimented at 400 g for 10 min; pellets were resuspended in HEPES-buffered Dulbecco's Modified Eagle's Medium (HDMEM, 4.5 g/l glucose; Sigma, St. Louis, MO), and cells were cultured into 25-cm^2 polyornithine coated tissue culture flasks (2.5 million cells/flask) in HDMEM containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. On day 3, the culture medium was replaced with HDMEM containing serum supplement (30 nM selenium, 20 nM progesterone, 1 mM iron-free human transferrin, 5 mM insulin, and 100 mM putrescin) and 1% penicillin/streptomycin. This medium was changed every 3 days until the release experiment.

Release studies

These studies were conducted after maintaining the MBH neurons in cultures for 8-9 days. The cultures were treated with 2 ml of serum-free chemically defined medium containing various doses (25, 50, 100 mM) of ethanol or no ethanol. In order to evaluate the changes of β-endorphin release (during a 3 h period) in the media following acute and chronic ethanol treatments, cultures were fed with fresh medium containing drug at 0, 3, 6, 21, 24 and 45 h, and the conditioned media from each culture were collected at 3, 6, 24 and 48h after the initiation of the treatment. The conditioned medium samples were collected into 12 **x** 75 mm glass tubes and stored frozen (- 20°C) until use for measurements of β-endorphin levels**.** At the end of the treatment, the cells from each culture were extracted and used for measurement of the total cellular protein level.

Protein measurement

The release of β-endorphin from the cultured MBH neurons was measured by determining the concentration of the peptide in the medium by enzyme immunoassay (EIA) using a kit purchased from Peninsula Laboratories, LLC (Torrance, CA). The assay was conducted according to the manufacture's protocol. The peptide release rate during a 3 h period of each culture was calculated using the media β-endorphin concentration (pg/ml) per μg of total cell protein, which was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA).

Statistics

The mean ± SEM of the data were determined and presented in the text. Data obtained in the studies dealing with ethanol time effects on β-endorphin release of each strain were compared using one-way ANOVA followed by Newman Keuls post-hoc test.

Ethanol time effects on β-endorphin release between *Per2* mutant and wild type mice were statistically evaluated using two-way ANOVA followed by Bonferroni ***t*** test. A value of $p < 0.05$ was considered significant.

RESULTS

Comparison of the effects of ethanol on β-endorphin release from MBH cells of control and Per2 mutant mice in cultures

Previous work using primary culture of cells of MBH of fetal rats has shown that β-endorphin release from these cells is elevated after acute (1-12 h) ethanol treatment, but decreased following chronic (24-48 hr.) ethanol treatment [54, 272, 273]. To evaluate the effect of *Per2* gene deletion on β-endorphin neuronal response to ethanol, the opioid secretory responses to various doses of ethanol at different time points were determined in cultured MBH cells of C57BL/6 or Per2 mutant mice.

As anticipated, MBH cells of control mice demonstrated significant β-endorphin-secretory response to acute ethanol treatments for a period of 3 and 6 h in a concentration-dependent manner (Fig.4). The effective doses of ethanol that were able to significantly elevate β-endorphin-release were within the range of 50 and 100 mM. However, MBH cells of Per2 mice failed to show the β-endorphin-secretory response to acute treatment with either of the ethanol doses (25-100 mM) studied. Interestingly, the basal release rate of β-endorphin from MBH cells of Per2 mice was significantly higher (2-5-fold) than MBH cells of control mice (Figs. 4 and 5).

When the effect of chronic ethanol treatment on β-endorphin-release of MBH cells of control mice was examined, the study revealed a significant inhibition of β-

endorphin-release at 24 and 48 h by all the doses (25, 50 and 100 mM) of ethanol without showing any dose response. Chronic treatment with any of these doses of ethanol failed to change β-endorphin-release from MBH cells of *Per*2 mice. This data suggest that *Per*2 deletion increases the basal release of β-endorphin neurons, but markedly alter the ability of this neuronal release to respond to ethanol.

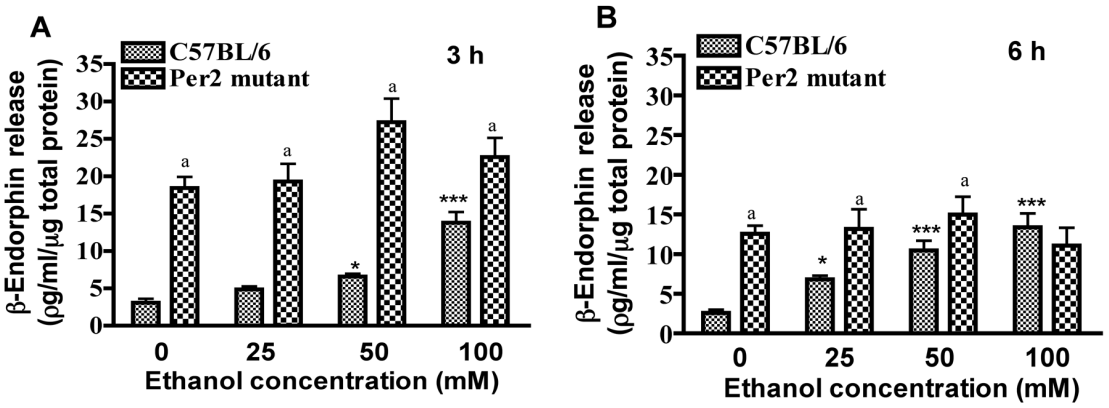


Figure 4. Effects of acute ethanol exposure on ß-endorphin release from mediobasal hypothalamic cells of C57BL/6 mice and *Per*2 mutant mice in primary cultures. MBH cell cultures were treated with (25, 50, 100 mM) or without ethanol for 3 hrs (A) or 6 hrs (B). Data are mean ± SEM of six independent observations. *P <0.05, ** P <0.01, ***P <0.001, significantly different from controls of the same strain. [a] P< 0.05, significantly different between two strains at the same dose.

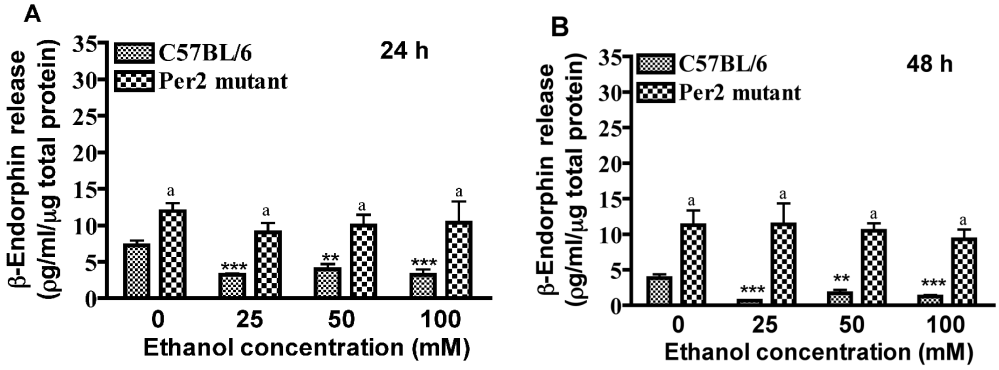


Figure.5. Effects of chronic ethanol exposure on ß-endorphin release in mediobasal hypothalamic cells of C57BL/6 mice and *Per*2 mutant mice. MBH cell cultures were treated with (25, 50, 100 mM) or without ethanol for 24 (A) to 48 hrs (B). Data are mean ± SEM of six independent observations. *P <0.05, ** P <0.01, ***P <0.001, significantly different from controls of the same strain. [a] P< 0.01, significantly different between two strains at the same dose.

Comparison of the effects of ethanol exposure on the tissue level of β-endorphin in the hypothalamus of control and Per2 mutant mice

The *in vitro* analyses of the effects of ethanol exposure on β-endorphin release from MBH cells of C57BL/6 or *Per*2 mutant mice clearly identified a targeted role of this clock-regulating gene in ethanol exposed. In the *in vitro* study, MBH cells in cultures were devoid of the influence of the rest of the CNS and peripheral systems. Hence, in vivo effects of ethanol on β-endorphin neuron were evaluated by measuring the changes in peptide levels in MBH following acute and chronic ethanol feeding.

Alcohol was administered using milk formula acutely (1 day) or chronically (5 days) in neonatal mice. When the MBH level of β-endorphin were compared between ad lib-fed and pair-fed groups after 1-day of feeding, no significant differences in the protein levels were found between these two treatment groups in C57BL/6 or in *Per*2 mutant mice (Fig. 6). The β-endorphin level in MBH was higher in acute alcohol-fed group than in ad lib-fed and pair-fed groups in C57BL/6 mice but not in *Per*2 mutant mice (Fig. 3). The peptide level in the MBH did not change after 5-day of milk formula feeding but was significantly reduced after alcohol feeding in C57BL/6 mice (Fig. 7). In *Per*2 mutant mice, the β-endorphin levels in MBH were similar in all groups. These results suggest

that, like in vitro, acute ethanol treatment increases while chronic ethanol treatment reduces the β-endorphin levels in MBH of C57BL/6 mice but not in *Per*2 mutant mice.

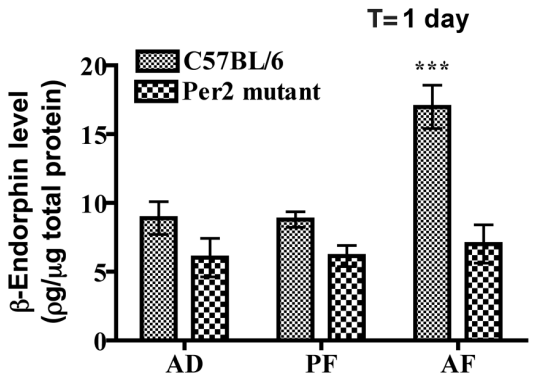


Figure 6. Effects of acute ethanol administration on ß-endorphin levels in mediobasal hypothalami of C57BL/6 and *Per*2 mutant mice. Postnatal mice were fed milk formula containing ethanol (AF) or no ethanol (PF) or left in the litter (AD) for 1 day. Data are mean ± SEM of six independent observations. *P <0.05, ** P <0.01, ***P <0.001, significantly different from controls of the same strain. [a] P< 0.01, significantly different between two strains at the same dose.

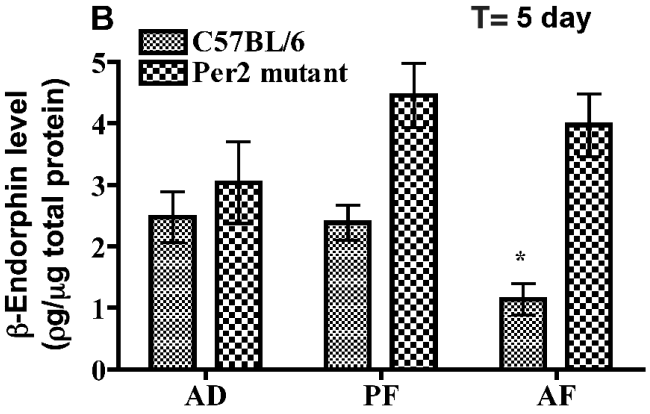


Figure 7. Effects of chronic ethanol administration on ß-endorphin levels in mediobasal hypothalami of C57BL/6 and *Per*2 mutant mice. Postnatal mice were fed milk formula containing ethanol (AF) or no ethanol (PF) or left in the litter (AD) for 5 days. Data are mean ± SEM of six independent observations. *P <0.05, ** P <0.01, ***P <0.001, significantly different from controls of the same strain. [a] P< 0.01, significantly different between two strains at the same dose.

DISCUSSION

In this article, we report for the first time the involvement of the *Per*2 gene in β-endorphin neuronal response to ethanol. We showed here that the *Per*2 mutation prevents the β-endorphin secretory response to acute ethanol and the β-endorphin inhibitory response to chronic ethanol treatments. Because the effect was observed not only *in vivo* but also *in vitro* cell culture systems where β-endorphin neurons lacked the influence of the central clock because of lack of the integrated neurocircuitry required fror suprachiasmatic nucleus (SCN) control mechanism, it could be suggested that the *Per2* gene regulates the internal clock to control β-endorphin neuronal response to ethanol. In support of this view is the data that prenatal ethanol alters Per2 and POMC mRNA levels in the arcuate nucleus of the adult rat hypothalamus [51]. Additionally, Per2 gene is identified in laser captured microdissected β-endorphin neurons [51].

We also demonstrated an increase in the basal release rate of β-endorphin in cells of MBH from *Per*2 mutants suggesting that the clock gene might have and inhibitory control over β-endorphin production and release. However, basal β-endorphin level in the MBH was similar in both C57BL/6 and in *Per*2 mutant mice, suggesting that clock gene inhibitory influence over β-endorphin production and release may be overcome by the stimulatory inputs from other CNS areas under the *in vivo* condition.

A core clock mechanism in the mouse SCN appears to involve a transcriptional feedback loop in which CLOCK and BMAL1 are positive regulators, and 3 *mPeriod (mPer)* genes are involved in negative feedback. Clock gene products BMAL1 and CLOCK have been shown to regulate circadian expression of several genes through their

binding to E-boxes [112]. Thus it is possible that the molecular circadian clock directly regulates β-endorphin producing POMC gene expression because there are putative E-box binding sites in the mouse POMC promoter [326].

Our data indicated a direct action of Per2 gene in regulation of POMC gene response to ethanol. This view is in agreement with the finding by Spanagel et al., who showed increased ethanol preference following Per2 mutation possibly because of altered excitatory neurotransmission and that the increase is not related to circadian timing [298]. Additional support for the involvement of *Per*2 gene in regulation of ethanol sensitivity comes from the indirect evidence showing that ethanol-induced suppression of immune function is correlated with the altered expression of *Per*2 gene in splenocytes [327] and m*Per*2 mutation disrupts circadian expression of cytolytic factors in splenocytes [55].

Another key question is the pathophysiological relevance of the circadian regulation of β-endorphin neurons, especially in the context of fetal alcohol spectrum disorders. β-Endorphin peptide and its precursor protein POMC control a wide variety of physiological functions, including stress control, feeding, immune functions, tumor control and positive reinforcement and motivational properties of alcohol [328-332]. Studies in laboratory rodents indicated that prenatal ethanol exposure produces significant alteration in circadian expression of *Per*2 and POMC genes in the hypothalamus [51], reduces immune function [310], increases the incidence of cancers [318], and increases alcohol-drinking behavior [298]. Mutations in *Per* genes in mice also has been shown to produce similar effects on β-endorphin neurons (Figs 4-7), immune system [55, 310, 311], cancer development [333] and alcohol drinking behavior [298]. Furthermore, the prenatal ethanol-induced stress axis dysfunction [14, 52] is associated

with abnormal expression of *Per*2 gene in β-endorphin neurons [51], which participate in stress control mechanism [52] [332]. Together these data provide support for the notion that the abnormality in *Per*2 gene function may participate in induction of many prenatal ethanol-induced pathophysiologies. Finally, much research is needed in order to further characterize the interaction between ethanol and *Per*2 gene, as well as the implications of this interaction for health.

CHAPTER 3

To evaluate the effect of *Period 2* gene deletion on postnatal alcohol programming of *POMC* and metabolic gene expression in the hypothalamus

INTRODUCTION

Numerous studies have reported health- and disease-related problems in offspring with fetal alcohol spectrum disorders (FASD). Indeed, offspring exposed to alcohol during fetal development have problems ranging from stress disorders [27, 334], altered metabolic functioning [38], impairment in the immune response [55, 310, 311], to disruptions in circadian rhythms [29, 51]. A critical component for the regulation of stress, metabolic, and immune functions is the *Proopiomelanocortin* (*Pomc*) gene [331, 332], which has been shown to be a target of alcohol and clock genes [51, 335]. Therefore, the resulting phenotypes of altered stress and metabolic responses owing to developmental alcohol exposure may be in part because of effects on POMC-producing neurons in the hypothalamus.

Once transcribed, the *Pomc* gene becomes a precursor for several bioactive peptides by posttranslational processing, including β-endorphin, adrenocorticotrophin and α-, β-, and γ-melanocyte stimulating hormones (MSH), which are involved in the regulation of food intake, metabolism, stress response [78, 336, 337], and immune regulation [338]. *Pomc* gene expression abnormalities are associated with obesity, hyperphagia, diabetes [188, 189, 339], and cancer [331]. Thus, POMC neurons are a key component regulating the metabolic signaling in the brain.

Within the hypothalamus, several key genes, including signal transducer and activator of transcription 3 (*Stat*3), ankyrin repeat and suppressor of cytokine signaling (SOCS) box-containing 4 (*Asb*4), sirtuin 1 (*Sirt*1), and peroxisome proliferator-activated receptor gamma coactivator1α (*Pgc*1α), are associated with *Pomc* function and play regulatory roles in metabolism. For example, *Stat*3 regulates *Pomc* gene expression [170], and the mutation of this gene is associated with a severe obesity phenotype [116]. *Sirt*1 is another key metabolic signaling gene that orchestrates adaptation to changing metabolic states in peripheral tissues [240]. Recently, *Sirt*1 has been found to localize in the arcuate nucleus of the hypothalamus and co-localizes with *Pomc*-producing neurons [241]. In the liver, both *Sirt*1 and *Pgc*1α peptides play a role to mediate the NAD+ mechanism. Interestingly, *Pgc*1α also co-localizes in *Pomc* neurons; however, the role that it plays in these neurons is unknown. Furthermore, gene expression levels of *Sirt*1 and *Pgc*1α are altered by alcohol exposure in the livers of adult rats [340]. Little is known about the function of *Asb*4 gene; however recent literature has indicated that *Asb*4 is expressed in the hypothalamic areas typically specific to regulating metabolic function. In *Pomc*-producing hypothalamic neurons, food intake regulates the gene expression level of *Asb*4 [231], thus indicating its importance in metabolic sensing in the brain.

Metabolic genes, in particular *Pomc* and *Sirt*1, are expressed in a circadian manner and are involved in core molecular clock function [51, 204]. Recently, clock genes have been connected to formation and progression of many diseases related to metabolic disorders [341, 342]. Clock genes, such as negative regulators, *Period* (*Period* 1, 2, and 3), and *Cryptochrome* (*Cry* 1 and 2), and positive regulators, *Clock* and *Bmal*1, act in 2 tightly coupled transcriptional and translational feedback loops that are able to

self-sustain a circadian rhythm [200]. Interestingly, it appears that *Per*2 gene is a putative target of alcohol and may be linked to metabolic disease. Initial support for this notion stems from the evidence showing that alcohol exposure in adulthood or during fetal development alters the circadian expression of *Per* genes in the hypothalamus and peripheral tissues [51, 55]. Additionally, *Per*2 gene mutant ($mPer2^{Brdm1}$) mice display enhanced alcohol consumption and preference [298], whereas $Per1^{Brdm1}$ mutant mice do not show such an enhancement in alcohol drinking behavior [313]. Evidence has shown alcoholics with a specific set of polymorphisms in the *Per*2 gene consume less alcohol than alcoholics without the polymorphisms [298]. Furthermore, under a metabolic disease state, the expression of *Per* genes is altered in peripheral tissues [343]. However, there are no data available that connect *Per* genes in the mediation of ethanol's (EtOH) programming of POMC-regulated metabolic functions. Therefore, we sought to determine whether postnatal alcohol exposure altered the expression levels of key metabolic genes in the hypothalamus of adult male and female mice.

MATERIALS AND METHODS

Animal Use

*Per*2 mutant ($mPer2^{Brdm1}$) and wild-type male and female mice of the same genetic background (C57BL/6J) were obtained from Jackson Laboratory (Bar Harbor, ME) and used in this study. $mPer2^{Brdm1}$ mice carry a mutant *mPer*2 gene with a deletion in the PAS dimerization domain, which is critical for interaction with other clock proteins [314], thus rendering a nonfunctional PER2 protein. Although the $mPer2^{Brdm1}$ mutant mice have an albino phenotype, because it was engineered with a tyrosinase gene deficiency that affects the ability to make melanin, the $mPer2^{Brdm1}$ mutant mice have the

same C57BL/6 genetic background with only a difference of the aforementioned gene deficiency. It has been shown that the albino mice strains 129/J, RF/J, SWR/J, AKR/J, A/J, and BALB/cByJ have a tyrosinase gene deficiency with no significant relationship between albinism and mean τ_{DD}, the endogenous (free-running) period of the circadian pacemaker measured in constant environmental darkness (reviewed in [335]). This suggests that the albino phenotype trait will have no implications on any circadian-related studies. Similarly, gradual EtOH exposure has been shown to produce an increase in alcohol preference both in C57BL/6 mice with normal tyrosinase activity and in BALB/cByJ mice with tyrosinase deficiency [344]. Furthermore, these 2 mouse strains (C57BL/6By and BALB/cByJ) showed no differences in their hypothermic response or the brain level of cGMP to the same EtOH dose [345]. These data support that the tyrosinase deficiency will have very little consequences in alcohol–response studies. *Per*2^{Brdm1} and C57BL/6 mice models have also been used previously in determining the role of *Per*2 in alcohol effects on the brain [249, 335].

*mPer*2 mutant and wild-type mice were maintained under constant environmental conditions on a 12-hour light/12-hour dark cycle (lighting period from 7:00 am to 7:00 pm) with ad libitum food and water. The *mPer*2^{Brdm1} mutant mice were routinely genotyped to verify the *Per*2 gene mutation. There were 4 primers used for detecting the *Per*2 gene wild-type and mutant variants. For the wild-type primer: forward-cttgggtggagaggctattc, reverse-aggtgagatgacaggagatc. For the mutant primer: forward-cattgggaggcacaagtcag and reverse-gagctgcgaacacatcctca.

Animal Treatments

C57BL/6 pups and *mPer2Brdm*[1] mutant pups (both sexes) were fed by intubation with milk formula containing either alcohol (alcohol-fed; AF) or an isocaloric volume of maltose dextrin (pair-fed; PF) as originally described previously [52], or kept in litter undisturbed (ad libitum-fed; AD). The alcohol–fed groups were given a milk formula containing EtOH (11.34% vol/vol; 0.1 to 0.2 ml/animal; during a period of 1 minute). The feeding was conducted at 10:00 and 12:00 hours from postnatal day (PD) 2 to PD7. After feeding, the pups were immediately returned to the litter. Some of these animals were killed 1 hour after the last feeding (13:00 hours) at PD7, the mediobasal hypothalami (MBH) were collected as previously described [51, 335] and immediately frozen for further analyses of various genes and proteins to determine the immediate effect of postnatal EtOH treatment on metabolic sensing in the MBH. The anogenital distance of postnatal mice at this age was too small to clearly identify the gender at this age and the sex of the experimental animals was not determined. Other animals were kept in the litter and weaned at 21 days of age. Female rats were ovariectomized bilaterally and subcutaneously implanted with a 0.5 cm estradiol-17β-filled silastic capsule (Dow Corning Corp., Midland, MI) under pentobarbital anesthesia. To clamp the estrous cycle changes of the steroid hormones [245, 346], we employed the procedure of ovariectomy and implantation of an estrogen capsule to maintain the animals in an estrogenic phase that is known to maintain the activity of POMC neurons in an elevated phase [347]. The capsule containing estradiol has been reported to maintain plasma levels about 75 to 100 pg/ml, similar to those observed during the preovulatory phase of the estrous cycle [246, 348]. The male and female rats were killed at PD90 at 10 pm, the brains were

dissected, MBH tissue samples were collected and immediately frozen for further analyses. Animal care and treatment were performed in accordance with institutional guidelines and complied with the National Institutes of Health policy. The Rutgers Animal Care and Facilities Committee approved this animal protocol.

β-Endorphin Immunoassays and Protein Measurement

The level of β-endorphin in the MBH tissue was measured by enzyme immunoassay using a kit purchased from Peninsula Laboratories, LLC (Torrance, CA). The assay was conducted according to the manufacturer's protocol. Total tissue protein was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA).

Real-Time Reverse Transcriptase Polymerase Chain Reaction Measurement

The total RNA was isolated from the hypothalamic tissue of each group (AD, PF, and AF) using the Trizol plus RNA purification system (Life Technologies, Grand Island, NY). Then, the high-capacity cDNA reverse transcription kit from Applied Biosystems (Foster City, CA) was used for the reverse transcriptase reaction. The complementary DNA was subjected to the real-time reverse transcriptase polymerase chain reaction on an ABI Prism 7500 sequence detector (Applied Biosystems). The Taqman probe primers (*GAPDH*, *Pomc*, *Per*2, *Stat*3, *Asb*4, *Sirt*1, and *Pgc*1α) were acquired from Applied Biosystems.

Statistics

Data are presented as mean ± SEM. The treatment effects and strain effects were determined by 2-way analysis of variance (ANOVA) with post hoc analysis using the Bonferroni post-test. $p < 0.05$ was considered significant.

RESULT

Comparison of the Effects of EtOH on POMC Neurons in Control and mPer2 Mutant Mice

In this study, we determined the changes in *Pomc* mRNA, β-endorphin and α-MSH levels at PD7 and PD90 in C57BL/6 and *mPer*2 mutant mice following administration of alcohol via milk formula or control treatments for 5 days. As shown in Fig. 8*A*1–*A*3, *Pomc* mRNA levels in PF and AD mice show similar levels at PD7 and PD90 in both C57BL/6 and *mPer*2 mutant mice. *Pomc* mRNA levels in the AF group were significantly lower than AD and PF groups on PD7 and on PD90 in both male and female C57BL/6 mice but not in *mPer*2 mutant mice. Mean levels of *Pomc* mRNA in control-treated C57BL/6 mice, in general, were higher than those in *mPer*2 mutant mice during the developmental period, although significant differences were only achieved between the PF groups on PD7 and AD groups (both male and female) on PD90.

β-endorphin levels in the MBH also did not differ between AD and PF groups at PD7 and on PD90 both in males and females (Fig. 8*B*1–*B*3). Comparison of the levels of β-endorphin between AF, PF, and AD mice shows that the level of β-endorphin was reduced in alcohol treated groups during the postnatal period (PD7) in C57BL/6 mice but not of *mPer*2 mutant mice. This alcohol effect remained till PD90 in both male and female of C57BL/6 mice. When the levels of β-endorphin were compared between 2 genotypes, it was observed that adult control-treated (AD and PF) C57BL/6 males had significantly higher levels of the peptide than those in *mPer*2 mutants, while AF-treated PD7 and PD90 C57BL/6 females had significant lower levels of β-endorphin than those in *mPer*2 mutant mice (Fig. 8*B*2),

The postnatal effects of EtOH on α-MSH levels in the MBH at PD7 and PD90 in C57BL/6 and *mPer*2 mutant mice are shown in Fig. 8*C*1–*C*3. The MBH tissue levels of α-MSH in AD and PF groups were similar at all time points in both C57BL/6 and *mPer*2 mutant mice. Comparison of the level of α-MSH between AF, PF, and AD mice indicate that the peptide level was lower in AF-treated animals on PD7 and on PD90 in both male and female C57BL/6 mice, but not in *mPer*2 mutant mice. When the endogenous levels of MBH α-MSH were analyzed between wild-type and *mPer*2 mutant mice, it was observed that, like the effect seen for β-endorphin, α-MSH levels were significantly different in adult control-treated males (*mPer*2 mutants had lower levels of protein) and AF-treated PD7 and PD90 females (*mPer*2 mutants had higher levels of protein).

The effect of postnatal EtOH treatment on mRNA levels of *Per*2 gene in the MBH tissues was also examined at PD7 and PD90 in both C57BL/6 and *mPer*2 mutant mice. Postnatal alcohol feeding suppressed *Per*2 mRNA levels during the postnatal period that persisted during the adult period in both male and female in C57BL/6 mice (Fig. 8*D*1–*D*3). Basal expression of *Per*2 mRNA did not differ at any developmental phase nor did it show any dimorphic effect when compared to wild-type mice (Fig. 8*D*1–*D*3).

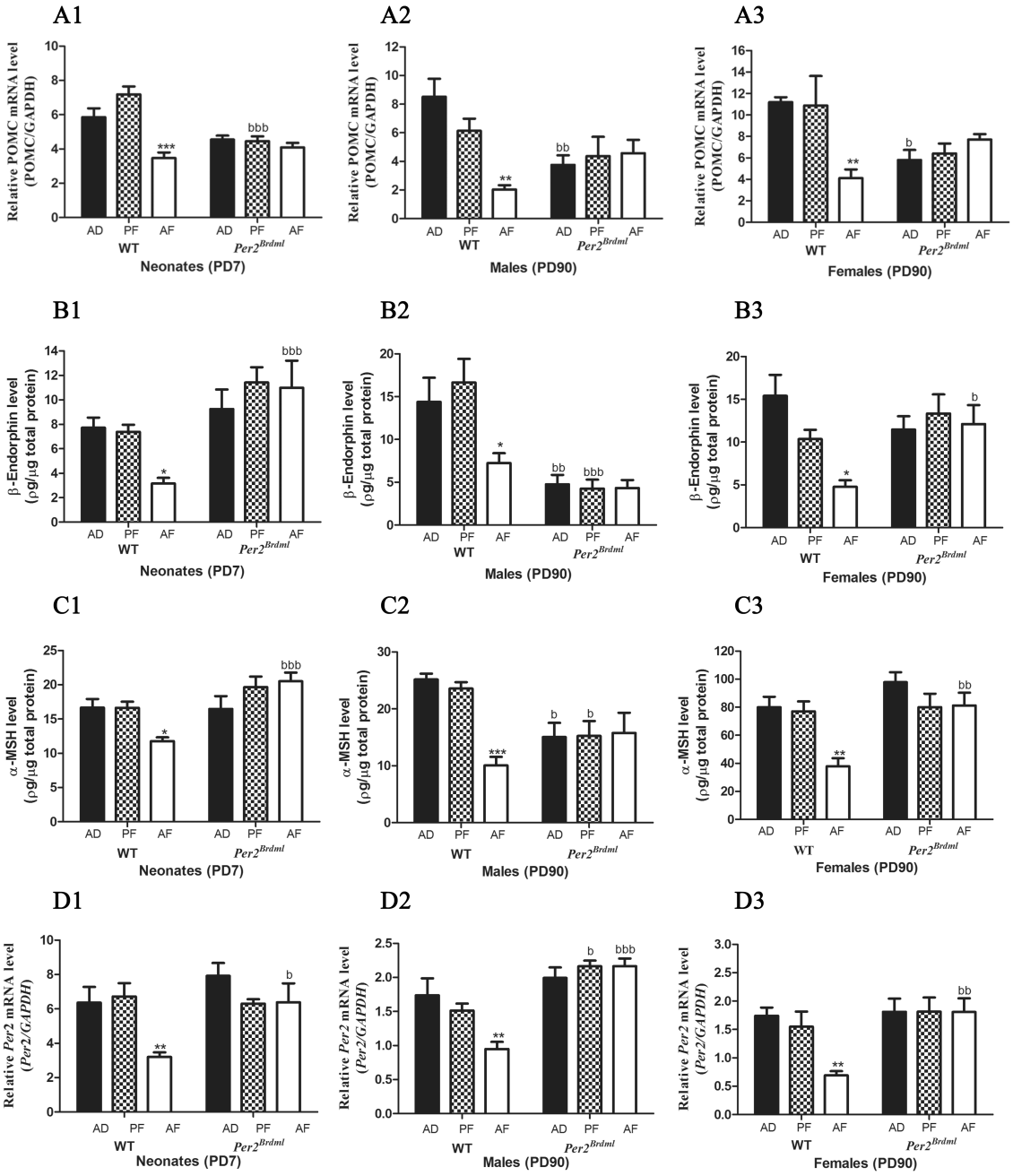


Figure 8. Effect of postnatal ethanol exposure on levels of proopiomelanocortin (*Pomc*) gene mRNA (**A**), β-endorphin (**B**), α-melanocyte stimulating hormone (α-MSH) (**C**), and *Per*2 (**D**) in the mediobasal hypothalamus (MBH) at PD7 and PD90 in C57BL/6 and *Per2Brdm*[1] mice. Pups fed with milk formula containing alcohol (AF), pair-fed isocaloric milk formula (PF), or left in the litter (AD) between PD2 and PD7. Data are mean ± SE.

$n = 6$. Two-way ANOVA identified significant interaction between feeding effects and genotypes ($p < 0.05$). Bonferroni post-test identified differences between control groups (AD and PF) versus AF-treated group within the same mice strain (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) or between C57BL/6 and *mPer*2 mutant mice in each feeding group ($^{a}p < 0.05$, $^{aa}p < 0.01$, $^{aaa}p < 0.001$).

Comparison of the Effects of Prenatal EtOH on the Expression of Metabolic Sensing Genes in the MBH of Control and mPer2 Mutant Mice

To test the role of *Per*2 in EtOH's action on the metabolic sensing of POMC neurons, we compared the effects of postnatal exposure to EtOH on mRNA levels of *Stat*3, *Sirt*1, *Pgc*1α, and *Asb*4 in MBH tissues of C57BL6 and *mPer*2 mutant mice at PDN7 and PD90. In general, the levels of all metabolic sensing genes at PD7 were higher than those at PD90. Postnatal alcohol feeding reduced all metabolic sensing genes in the MBH of both male and female C57BL/6 mice on PD7 and PD90. However, in *mPer*2 mutant mice, alcohol failed to produce any significant changes in the levels of most of these metabolic sensing genes on PD7 and PD90, with the exception of *Asb*4 mRNA levels on PD7 only. Genotype comparison revealed that on the postnatal phase on PD7, the mRNA levels of *Stat*3, *Sirt*1, and *Asb*4 in AD and PF groups showed a reduction in *mPer*2 mutant mice, compared with those in wild-type mice (Fig. 9). On PD90, AD and PF females showed no difference in the expression of all but the *Stat*3 gene between C57BL/6 and *mPer*2 mutant mice, while AD and PF males showed a reduction in *Stat*3 and *Asb*4 mRNA levels, and AD males showed a reduction in *Sirt*1 mRNA levels in *mPer*2 mutant mice than those in wild-type mice.

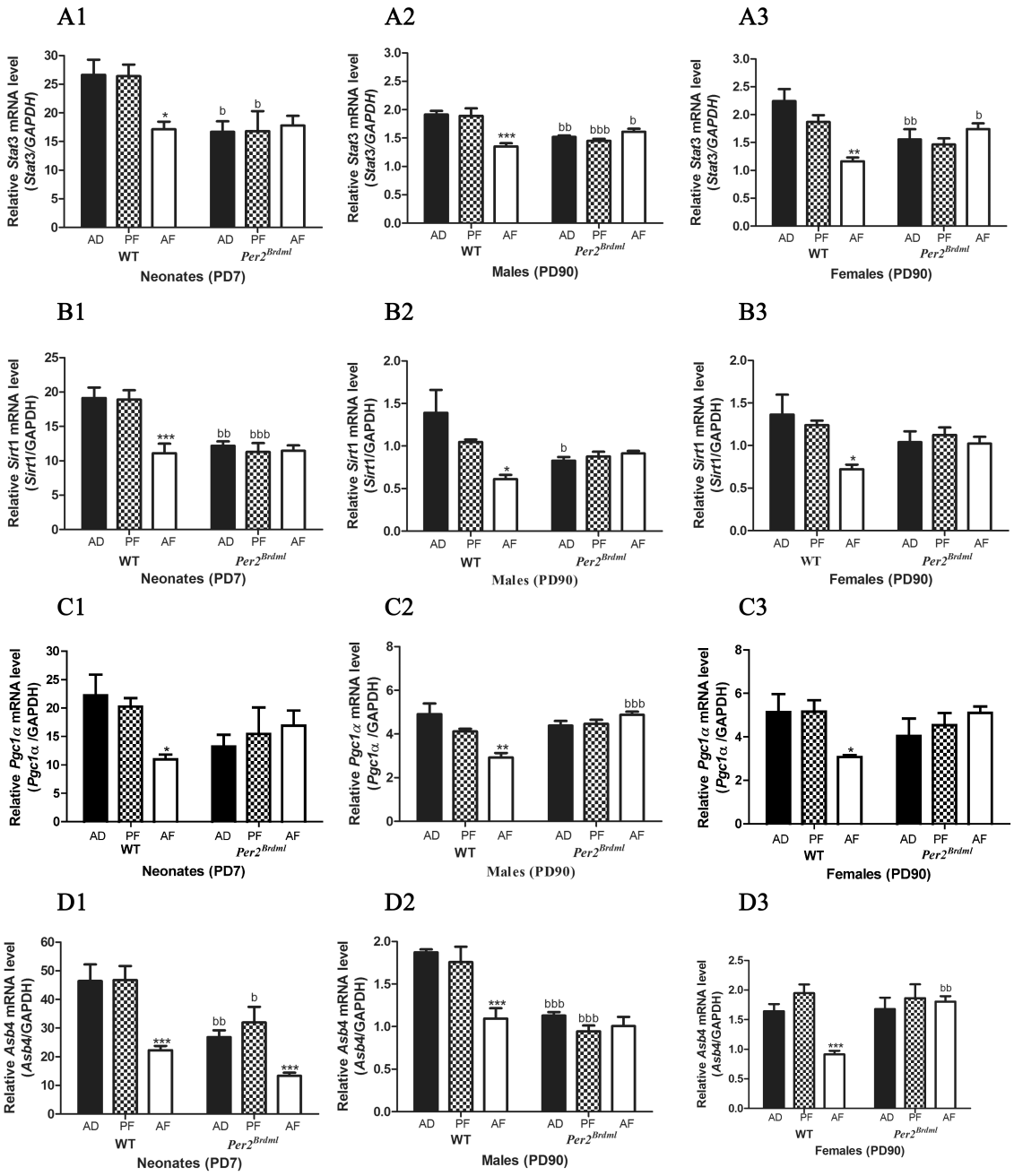


Figure 9. Effect of postnatal ethanol exposure on mRNA levels of *Stat*3 (**A**), *Sirt*1 (**B**), *Pgc*1α (**C**), and *Asb*4 (**D**) in the mediobasal hypothalamus (MBH) at PD7 and PD90 in C57BL/6 and *Per*2*Brdm*[1] mice. Pups were fed with a milk formula containing alcohol (AF), pair-fed isocaloric milk formula (PF), or left in the litter (AD) between PD2 and PD7. Data are mean ± SE. $n = 6$. Two-way ANOVA identified significant interaction

between feeding effects and genotypes ($p < 0.05$). Bonferroni post-test identified differences between control groups (AD and PF) versus AF-treated group within the same mice strain ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$) or between C57BL/6 and *mPer*2 mutant mice in each feeding group ($^{a}p < 0.05$, $^{aa}p < 0.01$, $^{aaa}p < 0.001$).

CONCLUSION

We report here that early-life exposure to alcohol significantly reduces the expression of *Pomc* gene and the production of β-endorphin and α-MSH peptides in the MBH that persists into adulthood. In addition, expressions of metabolic sensing genes in the MBH were also reduced as a consequence of postnatal alcohol exposure. Postnatal EtOH treatment also reduces the expression of one of the circadian clock genes *Per*2 that persists into the adulthood. Interestingly, in mice carrying a mutation of the *Per*2 gene and abnormal production of PER2 protein, the production of *Pomc* gene and its peptides as well as most of the metabolic sensing genes were reduced. Furthermore, alcohol exposure failed to induce further reductions in *Pomc* and metabolic genes and β-endorphin and α-MSH peptides in *mPer*2 mutant mice. Prenatal EtOH or *Per*2 mutation effects are found not to be sex-specific considering similar changes observed in both males and females in most of the cases. Because the actions of EtOH and *Per*2 mutation are in general similar on *Pomc* and metabolic sensing genes, and because *Per*2 mutation prevents EtOH to further alter metabolic gene expression, our data strongly suggest that *Per*2 may mediate EtOH's action on metabolic sensing genes.

Our findings on the involvement of *Per*2 gene in mediating EtOH's action on POMC neurons to alcohol are consistent with several previous indirect evidences. For example, Agapito and colleagues [335] have demonstrated that *Per*2 mutation prevented

β-endorphin stimulatory and inhibitory responses to acute and chronic EtOH challenges in a cell culture system. Also, prenatal EtOH decreases *Per*2 mRNA levels in the arcuate nucleus where many *Pomc* neuronal cells are localized [51]. Additionally, *Per*2 gene is identified in laser captured microdissected β-endorphin neurons [51], indicating that POMC-producing neurons express the *Per*2 gene. Also, a population of POMC neurons produces and releases glutamate [247], which is also a target of *Per*2 mutation[298].

How *Per*2 gene mutation alters EtOH's action on POMC-producing neurons is not well understood at present. One possibility is that the *Per*2 gene mutation leads to insufficient production of PER2 proteins leading to abnormalities in the clock mechanism governing POMC neuronal function. The other possibility is that PER2 is directly binding to the *Pomc* gene to alter EtOH's response. This concept seems somewhat heretical given the current paradigm that clock proteins inhibit expression by posttranslational modifications of the positive elements such as *Clock* and *Bmal*1 [196]. However, it is clear, at least in *Drosophila*, that PER is associated with DNA [202]. Moreover, recent studies in rat pituitary GH3 cells have shown PER proteins acting directly on the promoter of pituitary prolactin [349]. Therefore, one can assume that a similar process exists in other genes, including POMC.

The national center for disease control reported that approximately 34% of the U.S. population 20 years of age and over met the criteria for metabolic syndrome [198]. There is also a report that some fetal alcohol children show abnormal oral glucose tolerance tests with increased plasma insulin response [350]. The present data suggest that postnatal alcohol exposure in mice, equivalent to fetal alcohol exposure in humans, can be considered as a risk factor for developing metabolic-related disorders at later age.

Many studies have emerged to suggest that circadian processes are also critically involved in energy homeostasis [351, 352]. A number of studies have also connected the clock genes with metabolic sensing in the hypothalamus (reviewed in [197]). Association studies have revealed that shift workers, night workers, and sleep-deprived individuals with altered circadian clock mechanisms have an increased risk of developing symptoms of the metabolic syndrome [351]. In addition, altered sleep patterning has been implicated with abnormal leptin signaling, suggesting an implication of the circadian clock system in mediating metabolic signaling pathways in the central nervous system [199]. Furthermore, FASD patients are known to have altered sleep patterning [292, 353]. In view of this evidence, one can predict the involvement of the circadian clock in mediating metabolic sensing in the hypothalamus.

In this study, we demonstrated for the first time that EtOH-influenced expression of certain metabolic sensing genes (*Stat*3, *Sirt*1, *Pgc*1α, and *Asb*4) which appear to be regulated by *Per*2. All these metabolic genes were found to be express in *Pomc* expressing neurons, suggesting its symbiotic relationship with POMC in metabolic signaling in the hypothalamus. We postulate that developmental alcohol exposure may be altering the expression of the circadian clock genes, specifically in these neurons. This effect causes an alteration in the function of POMC neurons to mediate its metabolic signaling function by altering the expression of the other metabolism-regulating genes in the hypothalamus. More studies are necessary to determine how the *Per*2 gene mediates POMC neuronal functions and controlling EtOH action.

CHAPTER 4

Effect of Period 2 (*Per2)* gene deletion on postnatal alcohol Programming of circadian expression of metabolic genes in *Pomc*-expressing neurons

INTRODUCTION

Ethanol has been considered as a lifestyle factor that may influence the risk of type-2 diabetes mellitus. In healthy adults, binge ethanol consumption results in insulin resistance [354]. This is a particular public health concern because 7.6% of pregnant women and 51.5% of nonpregnant women reported drinking alcohol, and 1.4% of pregnant women and 15.0% of nonpregnant women reported binge drinking in the past 30 days [355]. Studies in rats showed that ethanol exposure during the prenatal and/or postnatal periods influences metabolic functions including increased gluconeogenic enzyme fructose-1,6-bisphosphatase [39], reduced insulin production and secretion [356], increased insulin resistance in the liver [357] and muscle [358] and impaired glucose tolerance by inducing insulin resistance and pancreatic beta-cell dysfunction [354, 359]. In addition, it has been shown that high-fat diet worsens glucose intolerance in offspring of rats exposed to ethanol [360] and chronic prenatal ethanol exposure increases adiposity and disrupts pancreatic morphology in adult guinea pig offspring [356]. It is not known whether the insulin resistance developed is secondary to altered body energy homeostasis as a result of, for example, an alteration in signaling in the hypothalamic proopiomelanocortin (POMC) neurons. Leptin and insulin, two anorectic hormones, have key roles in the regulation of body weight and energy homeostasis [361], as highlighted by the fact that several obese patients develop resistance to these hormones [362-364].

Within the brain, the hypothalamic proopiomelanocortin (POMC) neurons have been identified as one of the major targets of leptin and insulin actions [365] and play important roles in the development of obesity, insulin resistance and type II diabetes [366, 367]. Additionally, hypothalamic levels of a POMC derived peptide α-melanocyte stimulating hormone (α -MSH) has been shown to be lower in subjects with type 2 diabetes compared with controls [368]. These together with the evidence that early life exposure to ethanol suppress POMC gene expression and POMC-derived peptides beta-endorphin (BEP) and α-MSH production and secretion via an epigenetic programming [335, 369] led to the possibility that developmental alcohol may alter metabolic sensing of POMC neurons.

Another possibility is that early life exposure to ethanol alters the circadian mechanism governing POMC neuronal function to effect metabolic sensing of this neuron and thereby contributes to the metabolic dysfunction of the offspring. Recently, animal studies revealed that ethanol exposure during brain development causes alterations in the circadian regulatory system [46, 370, 371] and the circadian expression of POMC gene [50, 51]. The POMC system has been shown to play modulatory roles setting the level of induction of food-anticipatory activity rhythms by daily feeding schedules [372]. Given the dominant role of food as an entraining stimulus for metabolic rhythms [373], the circadian abnormality in POMC/melanocortin system may also be important in the pathology of metabolic system in fetal/postnatal alcohol exposed offspring. In this regard it is known that circadian clocks regulate metabolic homeostasis and clock disruption results in obesity and the metabolic syndrome [374-376].

Based on the aforementioned evidence of the teratogenicity effect of alcohol on the circadian system and the metabolic function, we tested whether alcohol exposure during the postnatal period alters the clock machinery to disrupt circadian expression of metabolic related genes present in POMC expressing neurons. We have chosen to disrupt circadian mechanism by gene mutation of period 2 (*Per*2), which is one of the negative elements of the molecular clock [377] [187] and is a target of ethanol [249, 335]. We have determined the circadian expression of a group of metabolic genes that are known to be involved in regulating insulin and leptin signaling in POMC neurons. We studied the expression profiles of silent information regulator T1 (SIRT1), peroxisome proliferator-activated receptor (PPAR)-γ co-activator 1α (PGC-1α) and ankyrin repeat and suppressor of cytokine signaling box-containing protein 4 (Asb-4) those act as downstream signaling of insulin receptor to regulate glucose homeostasis [243, 378-380]. We also determined the expression profiles of the signal transducer and activator of transcription-3 (STAT3) that mediate leptin signaling control of POMC neuronal functions [233, 381, 382]. We provide evidence here for the first time a role of circadian mechanism in fetal alcohol induced alteration of metabolic signals in POMC neurons.

MATERIALS AND METHODS

Animals:

Experiments were conducted using male mice, which were individually housed in 12h light/12h dark cycles (lights on at 07.00 hours, defined as Zeitgeber time ZT = 0). Animal care and treatment were performed in accordance with institutional guidelines, and were approved by the Rutgers Animal Care and facilities Committee and complied

with NIH policy. Adult C57BL/6J mice and *Per2* mutant (*mPer2*^{Brdml}) were obtained from Jackson Laboratory (Bar Harbor, ME). Transgenic mice expressing the fluorescent protein EGFP in POMC neurons (POMC-EGFP) were obtained from Dr. Malcolm Low. His laboratory generated these mice by introducing the EGFP cassette (fluorescent protein) into the 5' untranslated region of exon 2 of the mouse POMC genomic clone containing 13 kb of 5' and 2 kb of 3' flanking sequences [147]. The transgene was microinjected into pronuclei of one-cell-stage embryos of C57BL/6J mice (Jackson Laboratories). These mice are fertile and have normal growth and development [383]. The founder line had the expected distribution of positive cells based on the known pattern of POMC-expressing cells in the arcuate nucleus and in the nucleus of the solitary tract and pituitary melanotrophs and corticotrophs. *Per2* mutant (*mPer2*^{Brdml}) were crossed to POMC-EGFP transgenic mice to produce compound transgenic mice with mutant *Per2* in POMC cells (POMC-EGFP-*Per2*$^{-/-}$) mice. After sixth generations, transgenic homozygous POMC-EGFP-Per2$^{-/-}$ mice were obtained from different litters. These animals were genotype to determine their genetic allele. To determine the POMC-EGFP allele, the following primers were utilized: forward-TATATCATGGCCGACAAGCA; reverse-GAACTCCAGCAGGACCATGT. The polymerase chain reaction (PCR) cycling reaction conditions were the following: 94°C for 3 minutes, 35 cycles of 94°C for 30 seconds, 60°C for 1 minute, 72°C for 1 minute, 72°C for ten minutes and 4°C for 1 minute. The expected product size was 220 base pairs. To determine Per2 mutant allele, four primers were used: wild type primer; forward-CTTGGGTGGAGAGGCTATTC, reverse-AGGTGAGATGACAGGAGATC; mutant primer; forward-CATTGGGAGGCACAAGTCAG, reverse-

GAGCTGCGAACACATCCTCA. The PCR cycling reaction conditions were the following: 94°C for 3 minutes, 12 cycles of 94°C for 20 seconds, 64°C for 30 seconds, 72°C for 35 seconds, 25 cycles of 94°C for 20 seconds, 58°C for 30 seconds, 72°C for 35 seconds, 72°C for two minutes and 10°C for 1 minute. Two types different products were observed, the wild type allele (350 base pairs) or the mutant allele (280 base pairs). The transgenic POMC-EGFP-*Per2* $^{-/-}$ mutant mice contained a POMC-EGFP allele and a *Per2* mutant allele (280 base pairs). These animals were fertile and had normal growth and development.

Light:Dark (LD) or constant darkness(DD) treatment

In most studies, animals were maintained in 12 h light and 12 h dark cycle starting at 7:00 am for a minimum of 2 weeks. They were sacrificed at 4 h intervals during a 24h circadian period. Their brains were obtained and immediately frozen for RNA extractions and mRNA measurement or used for LCM collections of POMC neurons followed by RNA extraction and mRNA measurements. In some studies, the circadian nature of POMC and metabolic signaling genes were studied in POMC-EGFP mice after housing them in constant darkness (DD - 12h dark/12h dark cycles) environment for a period of two weeks. These animals were sacrificed at 4-hr intervals over a 24-hr period, the brains were collected, and the samples were stored at -80°C for further processing for LCM capturing of POMC neurons and gene expression assays by realtime (RT)-PCR.

Postnatal ethanol treatment

Both the transgenic POMC-EGFP (wildtype) and POMC-EGFP-*Per2*$^{-/-}$ mice were bred and their offspring were fed with ethanol containing milk formula or control diet between postnatal day (PD) 2 and 7. On the day of treatment pups from each litter were

divided into three groups and were given milk formula containing 11.34% ethanol (vol/vol) (AF) or isocaloric maltose (PF) or left in the litter with the mother (AD) as described previously [384]. The feeding was conducted at 1000 and at 1200 h daily for 5 days. After each feeding, the pups were immediately returned to the litter. Pups were remained with their dams till the weaning period at PD21. These animals were separated by sex and grouped housed in12 h light and 12 h dark cycle. At PD 90, the male offspring were sacrificed at 4-hr intervals over a 24-hr period, the brains were collected and stored at -80 C for gene expression studies.

Laser captured microdissection (LCM):

Brains were sectioned at 20 μm and processed for the dehydration steps. The slides were dehydrated in 75 % ethanol for 30 seconds, 95% ethanol for 30 seconds, 100% ethanol for 1 minute (twice) and xylene for five minutes. After these steps, the EGFP fluorescence containing POMC cells were viewed using a 488 nm filter and captured by using the PixCell LCM systems (Arcturus, Mountain View, CA, USA). Laser spot size 7 μm. The power amplitude and pulse duration of the PixCell laser were adjusted for each slide (65-75 mW, 750-850ms). The thermoplastic film-coated caps containing the captured cells were incubated in lysis buffer from the Picopure RNA isolation kit for 35 minutes (Molecular Devices; Mountain View; CA) and stored at -80 C for gene expression studies.

Total RNA extraction and RT-PCR:

The LCM-isolated POMC cells were processed for RNA isolation using Picopure RNA isolation kit and converted to cDNA by high capacity cDNA reverse transcriptase kit (Applied Biosystems; Foster city; CA). The cDNA yield was subjected to RT-PCR

using the specific 5' nuclease assay on an ABI Prism 7500 sequence detector (Applied Biosystems, Foster City, CA). The primers for POMC (Mm00435874_m1), STAT3 (Mm01219775_m1), Asb4 (Mm00480830_m1), Sirt1 (Mm01168521_m1), PGC1α (Mm00731216_s1), Per1 (Mm00501813_m1), Per2 (Mm00478113_m1), Per3 (Mm00478120_m1), Bmal1 (Mm00500226_m1), Clock (Mm00455950_m1), and GAPDH (4352932E) primers were acquired from Applied Biosystems. The RT-PCR was completed at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Relative quantity of mRNA was calculated by relating the PCR threshold cycle obtained from the tested sample to relative standard curves generated from a serial dilution of cDNA prepared from the total RNA. GAPDH mRNA in each sample was measured as an internal control for calibration by a control reagent. Relative mRNA levels in each time point were indicated as a percentage of the maximum value over the 24-h period of control group.

The statistical analysis:

Data were analyzed using one-way analysis of variance (ANOVA) with Dunnett post-hoc test to assess differences between the time points of the same group. In the Dunnett post-hoc test, the lowest value was chosen as the control. Two-way ANOVA with the Bonferroni post-test was used to assess differences between AD, PF and AF groups over the time. A *p* value less than 0.05 was considered statistically significant.

RESULTS

*Generation of the transgenic pomc-EGFP*Per2*^{Brdml}

To test the hypothesis whether the Per2 gene plays a role in mediating the pomc neurons, a transgenic mouse was created to contain both the *GFP-POMC* and *Per2*

mutant allele. Figure (10 a), shows the parental genotype analyses for GFP-POMC and $Per2^{Brdml}$ mice. Figure (10 b), demonstrated the transgenic offsprings *pomc-EGFP*Per2* mutant genotype that arose from the cross of both the GFP-POMC and $Per2^{Brdml}$ mice, these mice carries both genotype alleles (*gfp-pomc* and *per2 mutant*). Furthermore microscopic analyses shown in Figure (10 c – light microscope) and (10 d –fluorescent microscope) revealed that *pomc-EGFP*Per2* mutant mice contained *Pomc*-expressing neurons with the *GFP* marker in the arcuate nucleus of the hypothalamus. Figure (10 e) and (10 f) shows the isolated pomc expressing neurons in the LCM capsule under light and fluorescence microscopy. This capsule is used to isolate single cells via LCM technique and it facilitates the extraction of RNA from these cells. Figure (10 f) revealed that these neurons carried the GFP tag, suggesting that the neurons capture via LCM are specifically pomc-expressing neurons. Lastly, a model system to test the role of *Period 2* in *Pomc* expressing neurons was created.

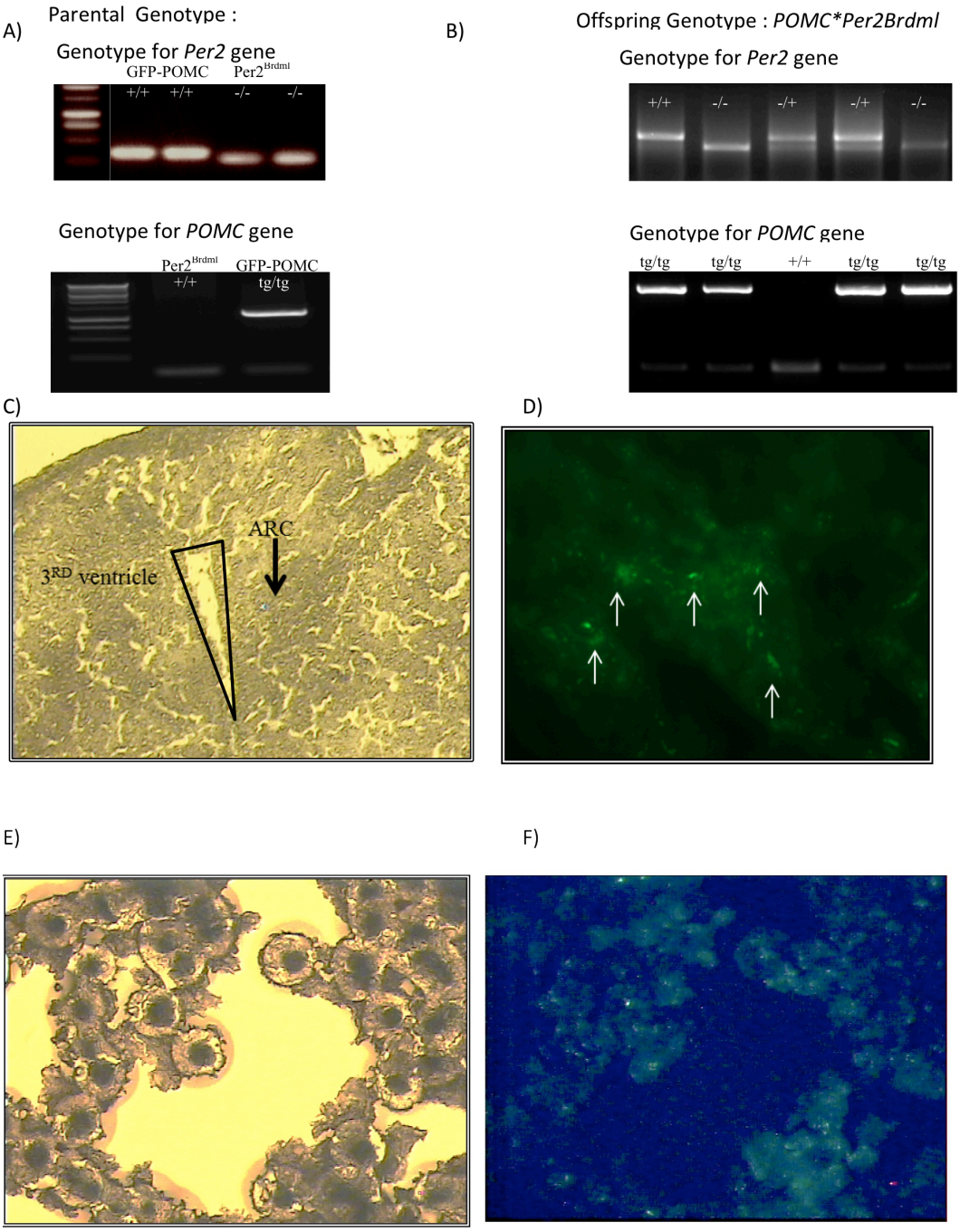


Figure 10: Generation of transgenic GFP-POMC*Per2^{Brdml}. (A) and (B) Genotype analysis of the parental and transgenic GFP-POMC*Per2^{Brdml} mice. (C) Brain section (20 µm) showing the arcuate nucleus of the hypothalamus under light microscopy. (D) Identification of POMC neurons (white arrowheads) by EGFP fluorescence. (E) POMC

neurons isolated using a LCM cap under light microscope. (F) POMC neurons isolated using a LCM cap under fluorescence microscope.

Proopiomelanocortin mRNA levels show circadian oscillation in the POMC neuronal population within the arcuate nucleus of the hypothalamus

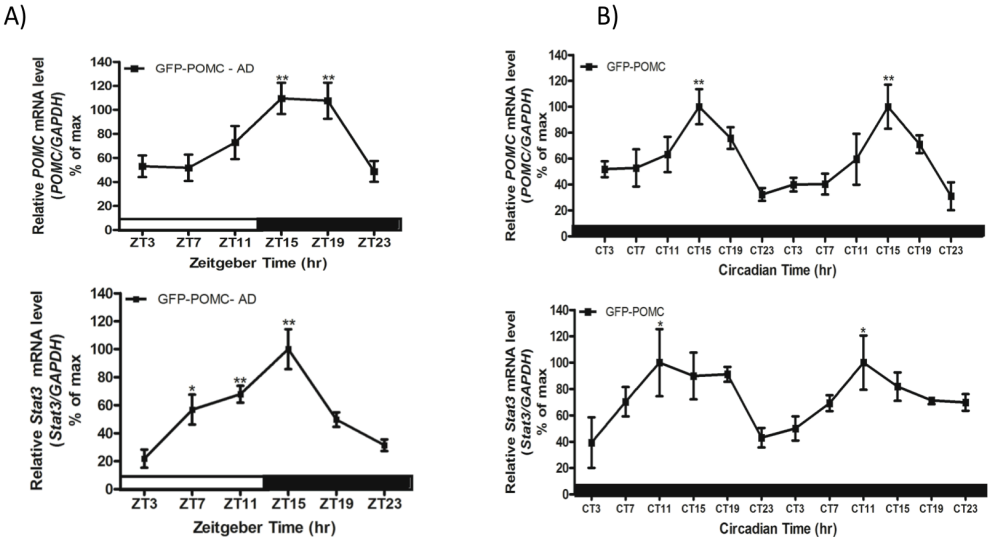
In rats, the daily rhythm of POMC mRNA in the arcuate nucleus [50] followed the rhythmic pattern of c-*fos* expression in POMC neurons [226, 385, 386]. In agreement with these findings, the data of present study show rhythmic expression of POMC mRNA in the arcuate nucleus ($p < 0.002$, Fig.11A) of C57BL/6J mice maintained in LD cycle. The POMC mRNA levels in this strain of mice were lower in daytime and started to increase from onset of the dark phase with the peak between ZT15 and ZT19 ($p < 0.01$), and then showed a gradual decrease until a nadir between ZT24 and ZT3. Like in C57BL/6J mice (data not published), the POMC mRNA expression in the LCM captured neurons of POMC-EGFP transgenic mice housed in LD cycle displayed the circadian variations over time ($p < 0.001$, Fig. 11A) with the identical rhythmic pattern. The daily POMC rhythm pattern in LCM captured POMC neurons did not change when the mice were housed in constant darkness suggesting that the POMC gene expresses in a circadian fashion in mice (Fig. 11B).

Stat3, Sirt1, Pgc1-α, Asb4 mRNA levels show circadian oscillations in POMC neurons in the arcuate nucleus of the hypothalamus

Energy- and food-reward homeostasis is the essential component for maintaining energy balance and its disruption may lead to metabolic disorders, including obesity and diabetes. If circadian functions of POMC neuron control body metabolism it would be expected to see circadian oscillations in the expression of insulin and leptin signaling

molecules in this neuron. To investigate this possibility, we performed relative mRNA measurements of a selective group of insulin and leptin signaling genes *Stat3*, *Sirt1*, *Pgc1-α*, *Asb4* by real time PCR in LCM captured POMC neurons obtained at different time points from POMC-EGFP mice housed under light/dark (LD) and constant darkness (DD) environmental conditions. Circadian oscillations of transcripts levels were found in all the signaling factors studied (Figure 11). It was observed that under LD conditions, rhythm peaks of *Sta3* and *Sirt1* were at a similar Zeitgeber time (ZT15). This time is during early night, which corresponds to the active state and initiation of feeding time in mice, reflecting their role in the metabolic function. Rhythm peaks of *Asb4* and *pgc1α* mRNA levels were at an earlier time period (ZT11) and were during the elevated phase cycle of their counterparts *Stat3* and *Sirt1* (Figure 11 A).

Under constant darkness, the mRNA level of *Pgc1α* mRNA shows it peak at CT15 and mRNA levels of *Stat3, Pgc1α,* and *Asb4* peaked at an advance time CT 11, CT 7 and CT3, respectively (Figure 11B). Since all the metabolic signaling genes showed rhythmic profiles under constant darkness conditions, these signaling molecules are also expressed in POMC cells in a circadian manner.



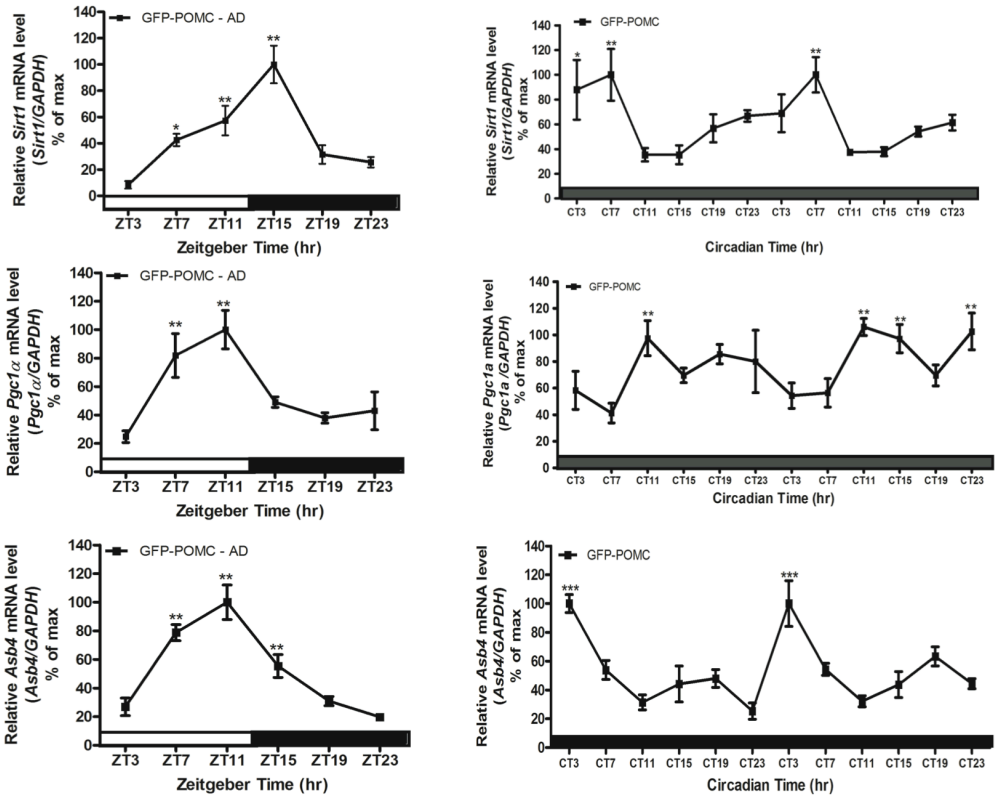


Figure 11: Daily rhythms in mRNA levels of metabolic related genes in pomc neurons of transgenic GFP-POMC mice under light/dark (LD) or 2 weeks constant darkness (DD) environmental conditions. (A) 24-h period (LD), or (B) 48-h period (DD). The isolated pomc cells corresponds to brains collected at 10:00 a.m., 2:00 p.m., 6:00 p.m., 10:00 p.m., 2:00 a.m., and 6:00 a.m. These time points corresponds with Zeitgeber times (ZT) 3, 7, 11, 15, 19, and 23, respectively. Relative quantification of mRNA levels was performed by real-time RT-PCR. mRNA levels were normalized with GAPDH values calculated as the percentage of the maximum value over the 24 h period. *, **, *** significantly different ($p < 0.05, 0.01, 0.001$) from the lowest value, as per one-way ANOVA with the Dunnett post-test. Black bar represent the dark period. Data are mean ± SEM of N= 5-6 animals per time point.

Early life exposure to ethanol disrupts circadian oscillations of POMC and metabolic genes in POMC neurons

In this study, we determined whether fetal ethanol exposure alters mRNA expression of POMC and metabolic signaling genes in POMC expressing neurons. Our results demonstrated that daily expression levels of *POMC, Stat3, Sirt1, Pgc1α, Asb4* mRNA show identical rhythmic profiles in both AD and PF animals, suggesting that the milk formula feeding method did not significantly alter the circadian expression of signaling and hormone genes in POMC neurons (Figure 12A). However, postnatal ethanol feeding using milk formula programmed the POMC neuron such that the adult expression of *POMC*, *Stat3, Sirt1* and *Asb4* mRNA levels became arrhythmic in this neuron. In addition, mRNA levels of these genes in AF mice were in general remained at the trough levels of the oscillatory states of these genes observed in AD and PF mice (Figure 12A). One exception is that *Pgc1α* mRNA levels in AF mice maintained oscillatory profiles. However, the peak of the *Pgc1α* mRNA oscillation in ethanol treated group showed an earlier peak at ZT7 as compared to AD and PF control group (Figure 12A). These data demonstrate that fetal alcohol exposure significantly alters the circadian expression of POMC and various metabolic sensing genes in POMC neurons.

Early life exposure to ethanol disrupts circadian oscillation of canonical clock genes in POMC neuron

We first examined whether POMC neurons present clock gene oscillations *in vivo*. For that purpose, we measured mRNA levels of a group of representative clock genes (Bmal1, Per1and Per2) that are known to participate in maintaining circadian rhythm in LCM captured POMC neurons at different time points in POMC-GFP mice.

The mRNA oscillations of the positive clock genes *Bmal1* peaked at ZT23 and showed the lower levels from ZT7 to ZT15 (Fig. 13A). These time points coincided with rising mRNA levels of *Per1* and *Per2*, their negative counterparts in the molecular clock. *Per1* mRNA levels peaked at ZT11 and were minimum from ZT19 to ZT23 (Figure 13A). *Per2* mRNA levels were found to be lower levels between ZT23 and ZT3, and were higher between ZT11 to ZT15 (Figure 13A).

We tested the possibility that alcohol targets the cellular clock mechanism to alter POMC neuronal functions. We first compared the mRNA levels of *Bmal1*, *Per1* and *Per2* in POMC neurons in control-fed (AD and PF) and alcohol-fed (AF) POMC-EGFP mice under LD environmental conditions. The circadian profile of *BMAL1, Per1*, and *Per2* in AD (Figure 13A) and PF (Figure 13A) groups showed no significance difference suggesting that the milk formula feeding had no significant effect on the circadian profile of these genes. However the circadian profiles of *BMAL1, Per1*, and *Per2* were quite different in AF group than those in AD and PF groups. Both the *Bmal1* and *Per1* mRNA levels in AF group displayed a significant lower levels during the peak circadian phase ($p < 0.05$) than in AD and PF groups (Figures 13A). Interestingly, the circadian profile for the Per2 gene demonstrated a robust difference in the rhythmic profile. *Per2* mRNA levels in AF group were lower at all time points than the AD and PF groups.

Additionally, *Per2* mRNA levels did not show any significant rhythm in POMC neurons of AF mice. This data demonstrates that early life exposure to alcohol alters the circadian profile of *Per2* and other key regulatory genes that maintain the cellular circadian clock machinery.

Per2 mediation of the effects of early life exposure to ethanol on circadian oscillations of POMC and metabolic genes in POMC neurons

Because fetal ethanol affect clock gene expression, the possibility is raised that circadian mechanism may involve in ethanol actions on POMC neurons. We first assessed if clock genes regulate circadian expression of POMC and metabolic signaling genes in baseline condition. To that end, we first determined whether Per2 gene mutation disrupts the cellular clock in POMC neurons. For this study we employed *Per2*^{Brdml} mice, which carry a mutant *mPer2* gene with a deletion in the PAS dimerization domain, which is critical for the interaction with other clock proteins, thus rendering a loss-of-function mutation of the PER2 protein.[314] We have previously shown that the expression of circadian clock genes in the spleen of *Per2* mutant mice was significantly distinct to that of wild type mice [310]. We crossed *Per2 mutant* mice with transgenic *POMC-EGFP* mice to generate the *POMC-EGFP-Per2*^{Brdml} mice. After genotype analysis the complex transgene showed both the *POMC-EGFP* and *Per2 mutant* allele (Figure 10).

In order to investigate the effects of *Per2* mutation on the molecular clock components in the POMC, we analyzed by real time RT-PCR the daily mRNA rhythms of *Per1*, *Per2*, and *Bmal1* in POMC neurons. Wild type mice displayed daily rhythms in *Per1*, *Per2* and *Bmal1* mRNA levels (Figure 13A). Consistent with previous reports describing clock gene expression in other mouse peripheral tissues,[387, 388] *Per1* and *Per2* peaked between ZT11 and ZT15, and *Bmal1* mRNA levels peaked between ZT23 and ZT3, maintaining its canonical antiphasic rhythm to *Per* genes (Figure 13A). Expression of circadian clock genes in the POMC neurons of *Per2* mutant mice was significantly distinct to that of wild type mice: *Per1*, *Per2* and *Bmal1* mRNA levels in the

Per2 mutant POMC neuron failed to display a significant daily rhythm (Figure 13B), which further emphasizes the regulatory role of *Per2* in controlling cellular circadian clock.[389] These results suggest that *Per2* mutation causes significant changes in the normal mRNA rhythms of key circadian clock genes in a specific population of neuronal cells. This can be envisioned as an overall defect in the molecular circadian clock physiology in the POMC neuronal population of *Per2* mutant mice.

We then analyzed the effect of Per2 mutation on postnatal ethanol effect on POMC and metabolic gene expression. Our analyses demonstrated that the major effect of *Per2* mutation on circadian expression of POMC and metabolic genes was that it prevented rhythmic expression of all these genes in AD, PF and AF mice (Figure 12B). Other subtle effects were that *Per2* mutation moderately shifted ethanol inhibitory effect on *Stat3* mRNA expression (Figure 12B). The mRNA levels of this metabolic signaling gene were significantly enhanced at ZT23, ZT3, ZT7, and ZT11 in AF mice as compared to AD and PF mice. The *Per2* mutation also increased *Sirt1* mRNA levels at ZT 19 and ZT23 but decreased the Asb4 mRNA level at ZT7 as compared to AD and/or AF group.

In summary, because the actions of postnatal ethanol and *Per*2 mutation are in general similar on POMC and metabolic sensing genes, and because *Per*2 mutation in general prevented ethanol to further alter metabolic signaling gene expression, our data strongly suggest that circadian clock may mediate ethanol's action on metabolic signaling genes and POMC in melanocortin neurons in the hypothalamus.

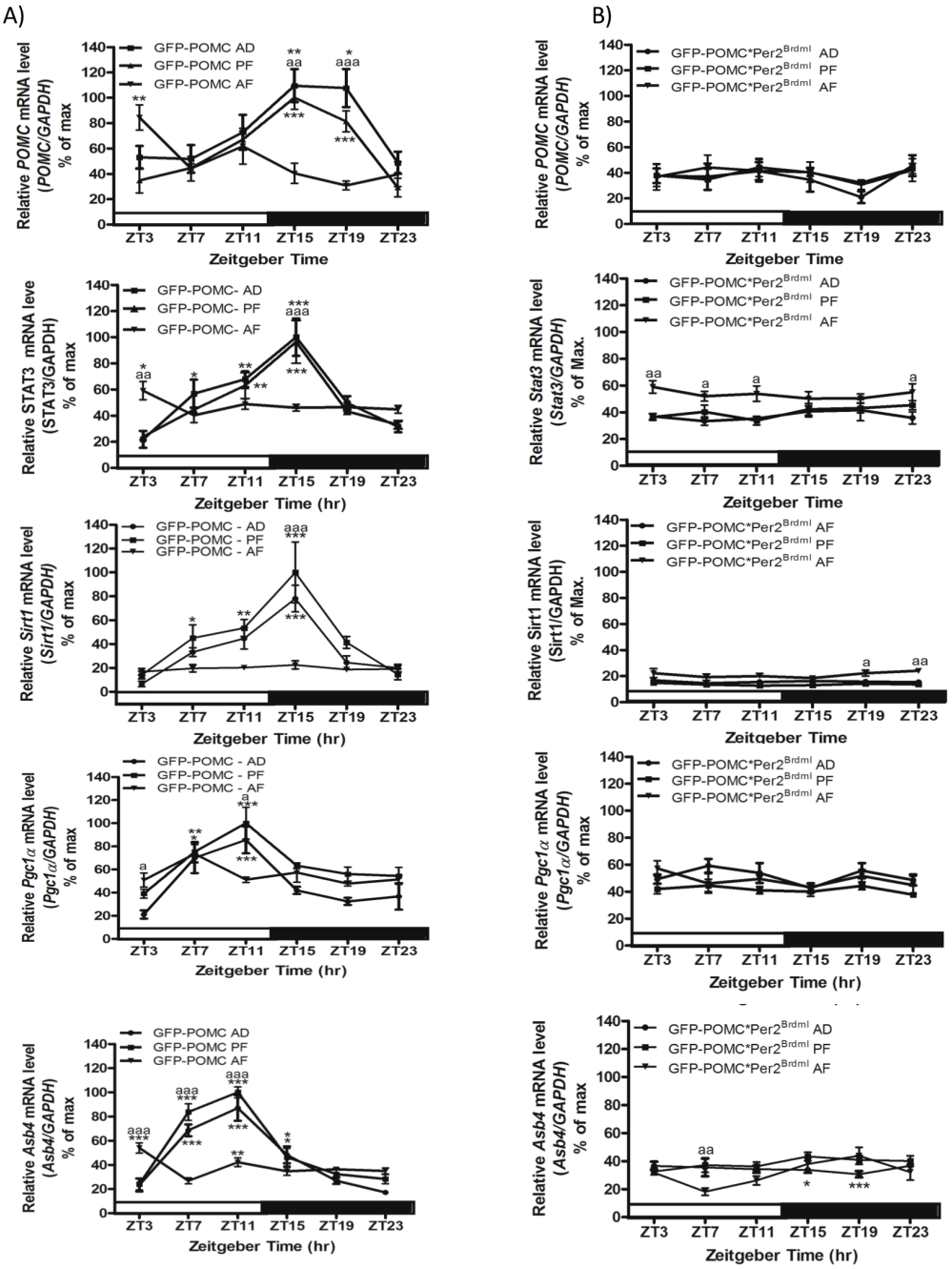


Figure 12: Daily rhythms in mRNA levels of metabolic related genes under ethanol or no ethanol treatments in pomc neurons of transgenic (A) GFP-POMC and (B) GFP-POMC*Per2^{Brdml} mice. Relative mRNA levels of *POMC, Stat3, Sirt1, Pgc1α,* and *Asb4* were calculated as the percentage of the maximum value over the 24-h period. *,**,*** Significantly different ($p < 0.05, 0.01, 0.001$) between means at different time points

among AD, PF and FAE groups from the lowest value, as per one-way ANOVA with the Dunnett post-test. [a, aa, aaa], significant difference ($p < 0.05, 0.01, 0.001$) between GFP-POMC and GFP-POMC*Per2^{Brdml} mice as per two-way ANOVA with the Bonferroni posttest. Black bar represent the dark period. Data are mean ± SEM of N= 5-6 animals per time point.

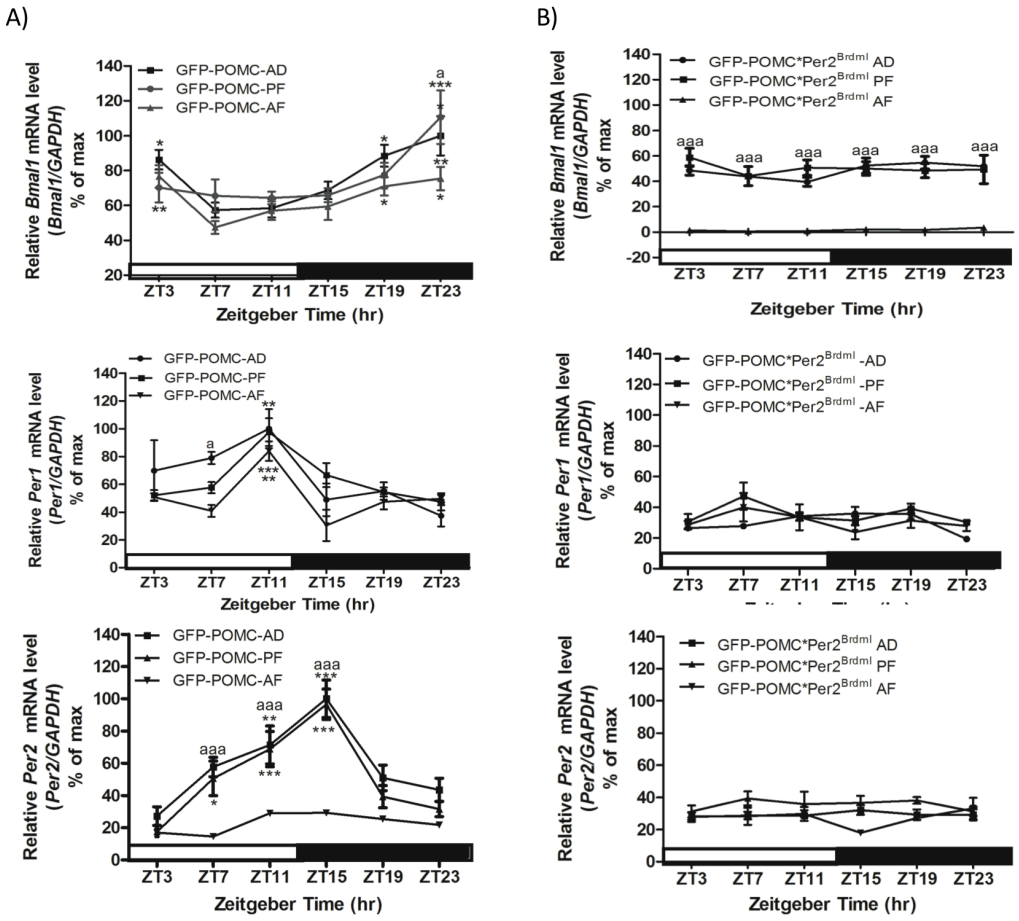


Figure 13: Daily rhythms in mRNA levels of clock genes under ethanol or no ethanol treatments in pomc neurons of transgenic (A) GFP-POMC and (B) GFP-POMC*Per2^{Brdml} mice. Relative mRNA levels of *Bmal1, Per1* and *Per2* were calculated as the percentage of the maximum value over the 24-h period. [*,**,***] Significantly different ($p < 0.05, 0.01, 0.001$) between means at different time points among AD, PF and FAE groups from the lowest value, as per one-way ANOVA with the Dunnett post-test. [a, aa, aaa], significant

difference ($p < 0.05, 0.01, 0.001$) between GFP-POMC and GFP-POMC*Per2^{Brdml} mice as per two-way ANOVA with the Bonferroni posttest. Black bar represent the dark period. Data are mean ± SEM of N= 5-6 animals per time point.

CONCLUSION

The national center for disease control reported that approximately 34% of the U.S. population over 20 years of age met the criteria for metabolic syndrome [198]. The present data suggest that postnatal alcohol exposure in mice, equivalent to fetal alcohol exposure in human, can be considered as a risk factor for developing metabolic related disorders at later age. Many studies have emerged to suggest that circadian processes are also critically involved in glucose homeostasis and liver physiology [351, 352, 390-392]. In addition, altered sleep patterning has been implicated with abnormal insulin and leptin signaling [393-397], suggesting an implication of the circadian clock system in mediating metabolic signaling pathways in the central nervous system [398]. Furthermore, FASD patients are known to have altered sleep patterning [292, 353] and metabolic problems [399-401]. In view of this evidence, one can predict the involvement of the circadian clock in mediating metabolic sensing in the hypothalamus. In this study, we demonstrated for the first time that the developmental ethanol induces changes in the expression of several metabolic sensing genes (*Stat*3, *Sirt*1, *Pgc*1α, and *Asb*-4) in POMC neurons that are regulated by a clock regulatory *Per*2 gene.

Previous studies have demonstrated that POMC neurons are regulated by nutrient-sensing and metabolic signals and are key component in metabolic homeostasis [402-404]. Mice with POMC neuronal abnormalities fed ad libitum exhibit normal to moderate

hyperinsulinemia, when subjected to a restricted protocol they develop hyperglycemia, glucose intolerance, and dyslipidemia [405]. Leptin, an adipose-derived hormone, conveys critical information about peripheral energy storage and availability to the POMC neurons. The anorexigenic actions of leptin are mediated by the long form of the leptin receptor, which activates both JAK2-dependent and -independent pathways, including the STAT3 pathway. In addition, leptin and insulin integrate with the central melanocortin system to coordinate alterations in energy and glucose balance. In the integration of leptin and insulin sensing various signaling molecules, including Asb4, Pgc1a and Sirt1, participate to control POMC neuronal function. All these metabolic genes are suggested to have symbiotic relationship with POMC in metabolic signaling in the hypothalamus. The data presented here suggest the possibility that developmental alcohol exposure alter the expression of the circadian clock genes to reduce the function of POMC neurons by altering the expression of the metabolism-regulating genes in melanocortin neuons.

Our findings on the involvement of *Per2* gene in mediating ethanol's action on POMC neurons to alcohol are consistent with several previous indirect evidences. For example, Agapito et al. have demonstrated that *Per*2 mutation prevented β-endorphin stimulatory and inhibitory responses to acute and chronic ethanol challenges in a cell culture system [335]. Also, prenatal ethanol decreases *Per*2 mRNA levels in the arcuate nucleus where many *POMC* neuronal cells are localized [51]. Additionally, *Per*2 gene is identified in laser captured microdissected β-endorphin neurons ([51]; Fig. 1), indicating that POMC-producing neurons express the *Per*2 gene. Also, a population of POMC

neurons produces and releases glutamate [247], which is also a target of Per2 mutation [298].

How *Per*2 gene mutation alters ethanol's action on POMC-producing neurons is not well understood at present. One possibility is that the *Per*2 gene mutation leads to insufficient production of PER2 proteins leading to abnormalities in the clock mechanism governing POMC neuronal function. The other possibility is that PER2 is directly binding to the *POMC* gene to alter ethanol's response. This concept seems somewhat heretical given the current paradigm that clock proteins inhibit expression by post-translational modifications of the positive elements such as *Clock* and *Bmal*1 [193, 194]. However, it is clear, at least in *Drosophila*, that PER is associated with DNA [202]. Moreover, recent studies in rat pituitary GH3 cells have shown PER proteins acting directly on the promoter of pituitary prolactin [349]. Therefore, one can assume that a similar process exists in other genes, including POMC.

In conclusion, the data presented here show that prenatal alcohol exposure suppresses Per2 gene expression and alters the function of circadian clock system to affect metabolic sensing and POMC production in melanocortin neurons in the hypothalamus. In this context it is interesting to note that specific PER2 polymorphism 10870 that reduces the function of these gene is evident in chronic alcoholic patients [293, 298, 406, 407]. Interestingly, *Per2* polymorphism contributed to changes in glucose metabolism, since Per2 SNIP was associated with metabolic syndrome [408]. Furthermore, perturbations of circadian clock components has shown to be associated with to islet pathophysiology in human type 2 diabetes mellitus [409]. Hence, we

postulate that FASD patients who have circadian abnormalities may also be susceptible to metabolic disorders.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

Four main conclusions can be drawn from the data presented throughout this manuscript: 1) *Per2* gene regulates the *Pomc* response to ethanol administration; 2) Neonatal exposure to ethanol significantly reduces the expression of *Pomc* gene and the production of β-endorphin and α-MSH peptides in the MBH that persists into adulthood; 3) *Per*2 may regulate and mediate ethanol's action on metabolic sensing genes (*Stat3, Asb4, Sirt1*, and *Pgc1α*) expressed in *Pomc*-expressing neurons; 4) Per2 may regulate the blood glucose response to ethanol exposure.

MODEL SYSTEM:

In order to develop a model system that implicates the participation of the opioidergic system, we need to consider mechanisms that would elucidate on how the *Per2* gene regulates *POMC*. In our study, we focus on four genes (*Stat3, Asb4, Sirt1*, and *Pgc1α*) that are co-expressed in *Pomc*-expressing neurons and are involved in metabolic function. By considering their role in peripheral tissues and assuming that such function may be detected in *Pomc*-expressing neurons, the following postulation can be formulated.

Humans and other species consume meals in discrete periods of time. Before a meal begins, the sensation of hunger initiates, which motivates the food-seeking behaviour. Once consumption begins, hunger declines (as the satiety signal is turned on). The term ‘satiation’ describes the processes that bring a meal to an end. An interval of time will then elapse before food consumption begins again. In this way, the body maintains a homeostasic environment. The main contributors of the satiety signaling in

the CNS are both insulin and leptin hormones, and their receptors are both expressed in the arcuate nucleus and have an inhibitory effect on food consumption [410, 411]. On the other hand, dieting or a state of nutrient deprivation leads to different signaling pathways that may involve SIRT1. It is widely speculated that the initiation of the SIRT1 signaling cascade results in the inhibition of the satiety signal and stimulates the appetite signal in the arcuate nucleus of the hypothalamus.

The satiety-signaling pathway driven by leptin is described as follows: The leptin hormone binds to the leptin receptor localized in *Pomc* expressing neurons. This leads to the phosphorlation of JAK2, which preempts the phosphorylation STAT3. The phosphorylated STAT3 then dimerizes and enters the cell's nucleus to regulate the transcription of the POMC gene. In the nucleus, phosphorylated STAT3 binds to the SP1 binding site in the POMC gene, which leads to an upregulation of POMC mRNA expression. This eventually leads to the release of αMSH, which inhibits food consumption (Figure 14).

In addition, there is a downstream inhibition of the leptin-signaling cascade, which is mediated by FoxO1. FoxO1 is a member of the forkhead box containing protein O superfamily. This protein is a central signaling molecule involved in many aspects of metabolism, including growth and proliferation as well as metabolic regulation through protein-DNA or protein-protein interaction [412, 413]. Fox01 exerts its inhibition in the leptin signaling mechanism via STAT3. In the nucleus, FoxO1 binds to the phosphorylated form of STAT3 and prevents it from interacting with POMC Sp1 binding site; as a result it inhibits STAT3 mediated leptin activation of POMC promoter [414](Figure 14).

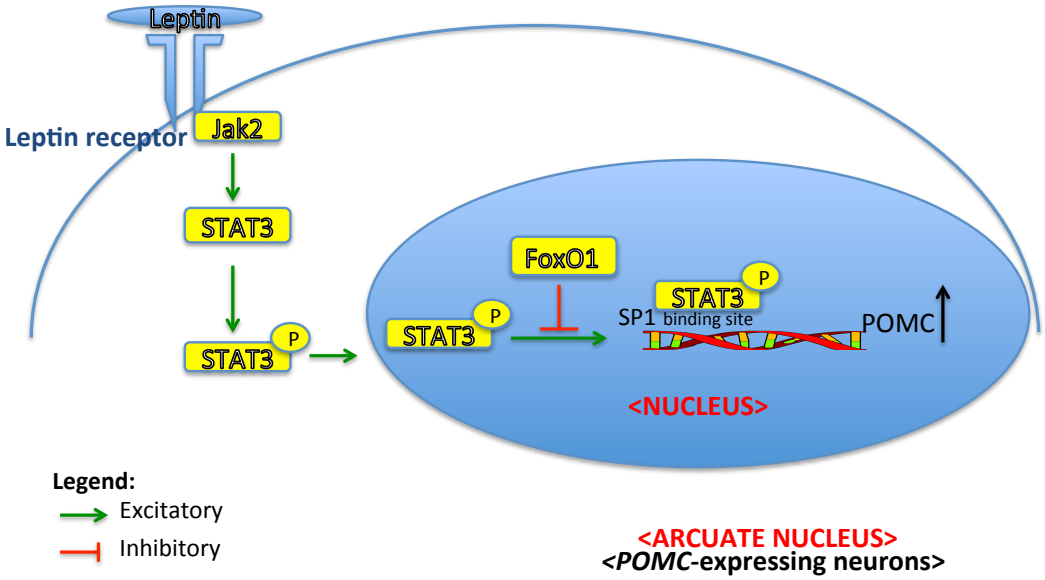


Figure 14. Leptin mediated signaling on POMC gene. These mechanisms demonstrate that the regulation of the opioidergic system via leptin signaling has both a stimulatory target through the phosphorylated form STAT3 and an inhibitory target mediated by the FoxO1 protein.

The insulin-signaling pathway in *Pomc*-expressing neurons is known to be a positive regulator of *Pomc* gene expression. FoxO1 is a downstream element of the insulin signaling pathway, studies have shown that when insulin binds to the insulin receptor, several intracellular substrates are recruited, including insulin receptor substrate (IRS) proteins. In the hypothalamus, both IRS2 and IRS4 proteins are expressed and it is thought that IRS4 inhibits IRS2 [415]. However both IRS4 and IRS2 are known to initiate the insulin-signaling cascade. These IRS proteins are phosphorylated by the activated insulin receptor, which is necessary to activate phosphatidylinositide 3-kinases (PI3K), leading to an enzymatic cascade that results in the activation of protein kinase B

(PKB, also known as Akt) [416]. Phosphorylation of FoxO1 by Akt results in its cytoplasmic shuttling from the nucleus, thereby inactivating FoxO1 as a transcription factor [378]. This in turn leads to two outcomes: 1) It abolishes the inhibition effect of FoxO1 on the POMC gene, and 2) it stimulates the production of α-MSH by relieving the interaction of FoxO1 with carboxypeptidase E (Cpe) [417]. FoxO1 regulates the expression of Cpe, which is one of the peptidases required for the processing of POMC into α-MSH [417] (Figure 15).

Interestingly, it has been shown that ASB4 blocks the insulin-signaling cascade via IRS4, which results in FoxO1 not getting phosphorylated [231]. This prevents phosphorylated STAT3 from binding to the Sp1 binding site at the POMC gene, thus inhibiting POMC gene expression. Additionally, the evidence suggests that (under a fed state) the Asb4 mRNA levels increase whereas (under fasting state) the Asb4 mRNA levels decrease in *Pomc*-expressing neurons. This further indicates that ASB4 may serve as a relay component to the opioidergic system under certain conditions (Figure 15). The signal cue that drive this condition remains to be elucidated.

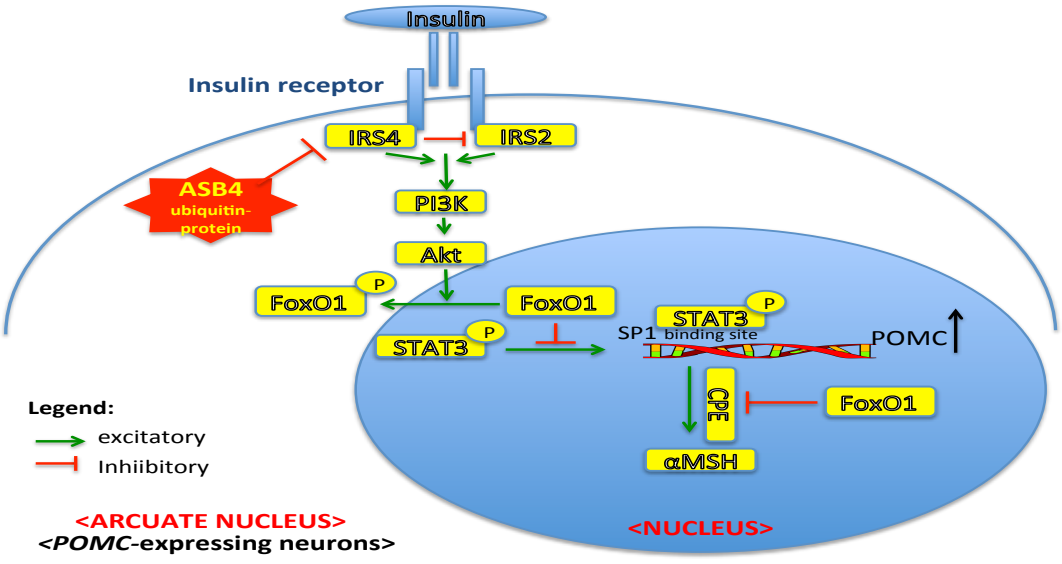


Figure 15: The effect of Asb4 as a negative regulator of the POMC gene via the insulin mediated mechanism. ASB4 inhibits IRS4 leading to inhibition of the Akt effect on FoxO1. This results in downregulation of *Pomc* gene expression.

In the hypothalamus, the nutrient mediated mechanism is activated under restricted dietary condition. Studies indicate that SIRT1 may in part drive this signaling cascade. In peripheral tissues SIRT1 protein expression levels increase under elevated NAD+ concentration, enhanced hepatic glucose output, enhanced gluconeogenic gene expression, enhanced *Nampt* gene expression, or fasting conditions. Thus we speculate that SIRT1 may serve the same metabolic function in pomc expressing neurons under nutrient deprivation conditions

In our model, we propose that under restricted feeding conditions, an unknown SIRT1 (X) activator will activate SIRT1 resulting in the upregulation of SIRT1 in POMC expressing neurons. In turn, SIRT1 will deacetylate both PGC-1-α and FOXO1 resulting in their activation. The activation of both PGC1-α and FoxO1 will lead to the suppression of POMC gene expression via leptin mediated signaling [241] or via carboxypeptidase E [417]. It is suggestive that both PGC1-α and FoxO1 work together to downregulate the effects of POMC gene. However the true role of neuronal PGC1α in pomc-expressing neurons is unclear (Figure 16).

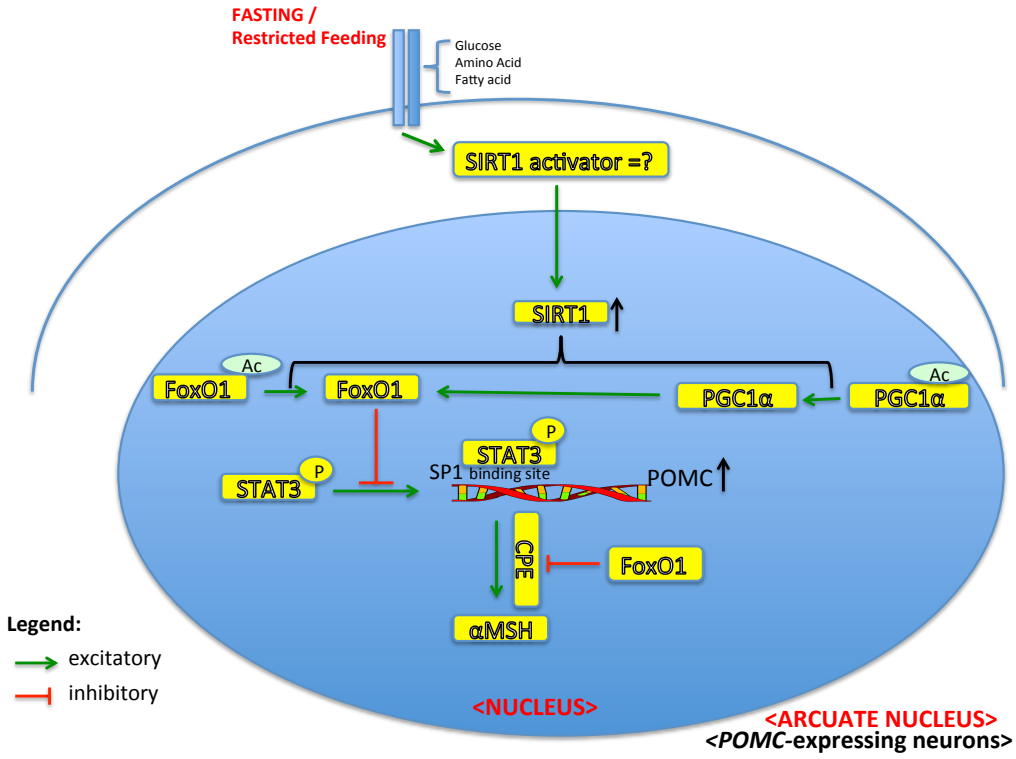


Figure 16: SIRT1 inhibition effect on the POMC gene. SIRT1 activates both FoxO1 and PGC1α. Their activation leads to downregulation of Pomc gene and α-MSH.

Our analysis reinforces previously published data and lead us to propose that *Stat3* and *Per2* may be positive regulators whereas *Asb4*, *Sirt1* and *Pgc1α* genes may be negative regulators of the *Pomc* gene transcription.

Our results suggest that under a 24 hr. daily cycle, PER2 may be the timekeeper for the *Pomc* gene in the arcuate nucleus of the hypothalamus. We have shown that in adult PER2 deficient mice the *Pomc* mRNA expression levels were reduced and gene rhythmicity was lost (chapter 2 and 3). In addition, *Pomc*-expressing neurons from the PER2 deficient mice demonstrate reduced expression of metabolic related genes (*Stat3, Asb4, Sirt1*, and *Pgc1α)* and loss of circadian rhythmicity of these genes. As a result, we propose that PER2, a circadian clock component, may mediate the expression of (*Pomc*,

Stat3, Asb4, Sirt1, and *Pgc1α)* in *Pomc*-expressing neurons. The stimulus that drives PER2 to mediate the expression of these genes remains to be elucidated (Figure 17).

The mechanism by which PER2 interacts with the *Pomc* gene to mediate its expression remains to be elucidated. Two speculations can be elucidated: 1. Abnormal PER2 production may lead to altered regulation of the clock mechanism governing POMC neuronal function. 2. Recent studies by Yoshitane and Bose have given evidence that Period proteins in both drosophila and pituitary GH3 cells can directly interact with DNA structures [202, 349]. Hence, PER2 mediation may be via an interaction with the promoter sites of these genes (*Stat3, Asb4, Sirt1*, and *Pgc1α*) to control *Pomc* gene expression control or via a direct interaction of PER2 protein on the *Pomc* gene. One can hypothesized that PER2 protein may bind to the *Pomc* E-box binding sites and drive its promoter, all these speculations remains to be elucidated.

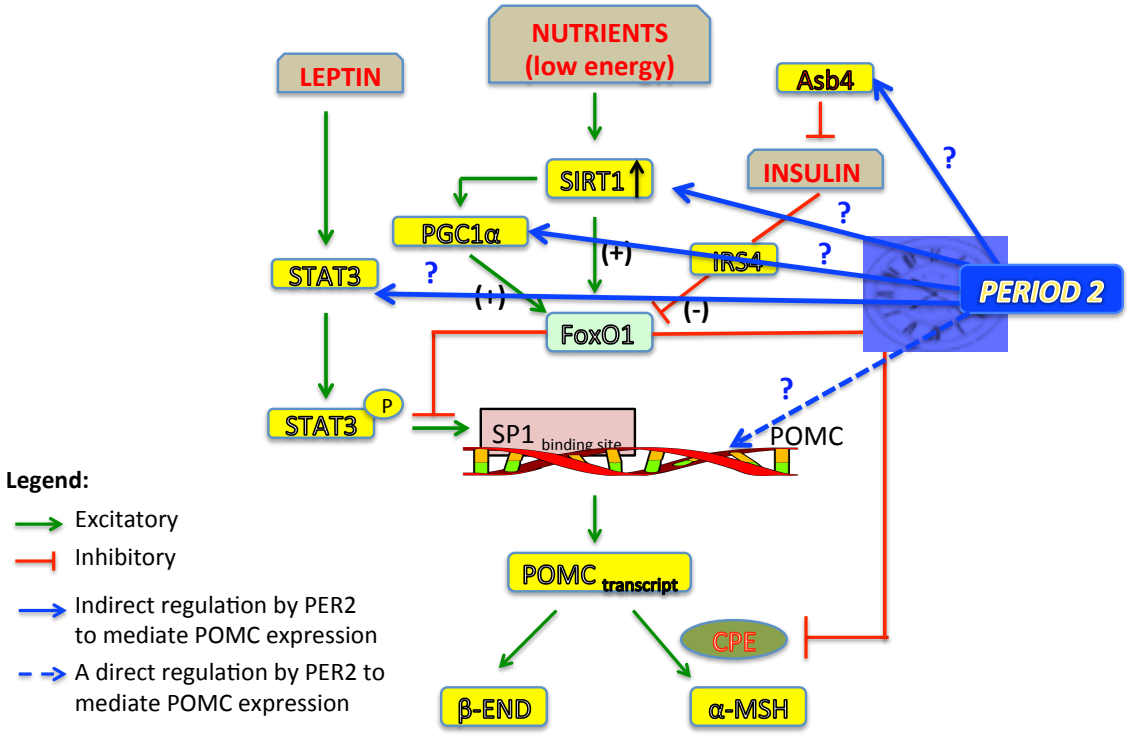


Figure 17. A proposed model for *Pomc* gene regulation by PER2. PER2 may mediate the *Pomc* gene in two ways: it can exert its regulation directly on Pomc gene or indirectly by influencing other key metabolic genes such as *Stat3, Asb4, Sirt1*, and *Pgc1α*.

Previous studies and our current data suggest that PER2 is a metabolic target for ethanol. We described that the C57BL/6 ethanol treated mice exhibited altered rhythmic profiles and reduced gene expression of *Pomc, Stat3, Asb4, Sirt1*, and *Pgc1α*. In addition, ethanol treated PER2 mutant mice showed no ethanol response, and the gene expression level were similar to the C57BL/6 ethanol treated mice. These observations imply that PER2 may mediate the gene expression and the response to ethanol of *Pomc, Stat3, Asb4, Sirt1*, and *Pgc1α* genes.

Our data observed (as described in chapter 3) clearly shows that postnatal ethanol exposure results in the downregulation of *Pomc, Stat3, Asb4, Sirt1*, and *Pgc1α* gene expression. We are not the only group that has shown this gestational alcohol effect, Hard's group conducted a study in mice and identified 75 genes that were downregulated by exposure to alcohol on days 7 and 9 of fetal development, but none of these were upregulated. For the most part these genes played a role in cell proliferation, differentiation, and apoptosis [418]. Our analysis differs in that the genes studied in this manuscript are mainly responsible for cell survival and metabolism.

The following speculation has been proposed to describe the action of ethanol on PER2. It is important to reemphasize that POMC neurons appear as early as embryonic day 12, but their projection pathways are developed after birth. With the same concept applied in the SCN, the clock genes (Per1, Per2, Bmal1, Clock and Cry1) initiate their

expression at embryonic day 19 without SCN rhythms. It is after birth that the SCN rhythms are initiated and established. Since our model demonstrates that ethanol either disturbs or abolishes the rhythms of the *Per2,* clock gene and metabolic related genes in *Pomc* expressing neurons, it can be suggested that ethanol may target POMC expressing neurons by altering the maturation of both metabolic and circadian signaling in POMC expressing cells. This effect may lead to lack of circadian coordination that eventually yields a metabolic signaling cue disruption in Pomc-expressing neurons as shown in (Figure 18).

In addition, we speculated that ethanol acts on PER2 and disrupts its circadian interaction with key metabolic genes (*Pomc, Stat3, Asb4, Sirt1*, and *Pgc1α),* which are expressed in *Pomc*-expressing neurons. This results in the reduction of expression levels and the loss rhythmicity or shift in the circadian profiles of these genes (Figure 18). This effect may result from ethanol disruption on cAMP synthesis in Pomc-expressing neurons. Evidence for this has been shown by Saffrey's lab that demonstrated that chronic exposure to ethanol alters the synthesis of cAMP in the brain [419]. Moreover, the lacked of cAMP synthesis may interrupt the CREB signaling pathway that may result in reduction in CREB expression or inhibition in CREB phosphorylation. Previous studies have shown that ethanol impairs phosphorylation of CREB. For example, Yang's lab demonstrated that neuroadaptation to chronic ethanol exposure includes alterations in CREB that may disturb gene expression that depend upon CREB for transcriptional activation [420]. Another study led by Li's lab showed that chronic ethanol administration reduced phosphorylation of CREB [421]. More interestingly is the finding from Lee's lab that demonstrated that at the cellular level in the SCN, reduction of CREB

expression as observed in CREB repressor mice resulted in diminish protein expression of PERIOD1 and PERIOD2 [422]. Suggesting that a similar paradigm can be observed in other cellular clocks. Our model proposes that alcohol may act on cAMP thereby altering the CREB signaling pathways resulting in reduction of PERIOD2 expression. In turn this effect can lead to reduction in *Pomc* gene expression. More investigation needs to be accomplished to verify this proposal.

Prospective investigations should be the following: How does ethanol interact with cAMP, CREB, PKA in Pomc expressing neurons in wildtype and in other clock related mutant mice? In this manner, we can compare whether the circadian clock drives this effect or whether it is a secondary function of a component of the circadian clock system that is not mainly driven by the circadian clock system.

The second question that can be proposed may be how does PER2 mediate *Pomc* gene expression? In our model, we propose that this mediation may be in three ways: it can be driven via transcription/posttranslational manner directed by the clock genes, since it was shown that the *Pomc* gene expression is driven in a circadian manner. Second, *PERIOD2* may have a dual function in regulating *Pomc* gene expression under certain conditions such as under ethanol exposure (as shown in our thesis manuscript data). This mechanism that drives this effect needs to be elucidated. Lastly, PER2 may be associated directly with *Pomc* gene or indirectly via an interaction with other metabolic regulatory genes such as *Stat3, Asb4, Sirt1*, and *Pgc1α* genes. This stipulation may seem impossible since it is known that clock regulatory proteins interact with other proteins in a posttranslational manner, However new evidence have suggested that PERIOD proteins may directly interact with DNA [202, 349]. This finding give rise to the following

presumptions: If it is a direct interaction, we need to identify the binding site of PER2 on the POMC gene. We have predicted that PER2 may directly bind to *Pomc* via E-box binding sites and drive its promoter. It is also possible that PER2 may mediate *Pomc* gene expression via an indirect mechanism by influencing the expression levels of *Stat3, Asb4, Sirt1*, and *Pgc1α* genes. Therefore, it will be important to identify PER2 binding siteson these metabolic genes and locate the regulatory binding sites of each of these metabolic proteins for the POMC gene.

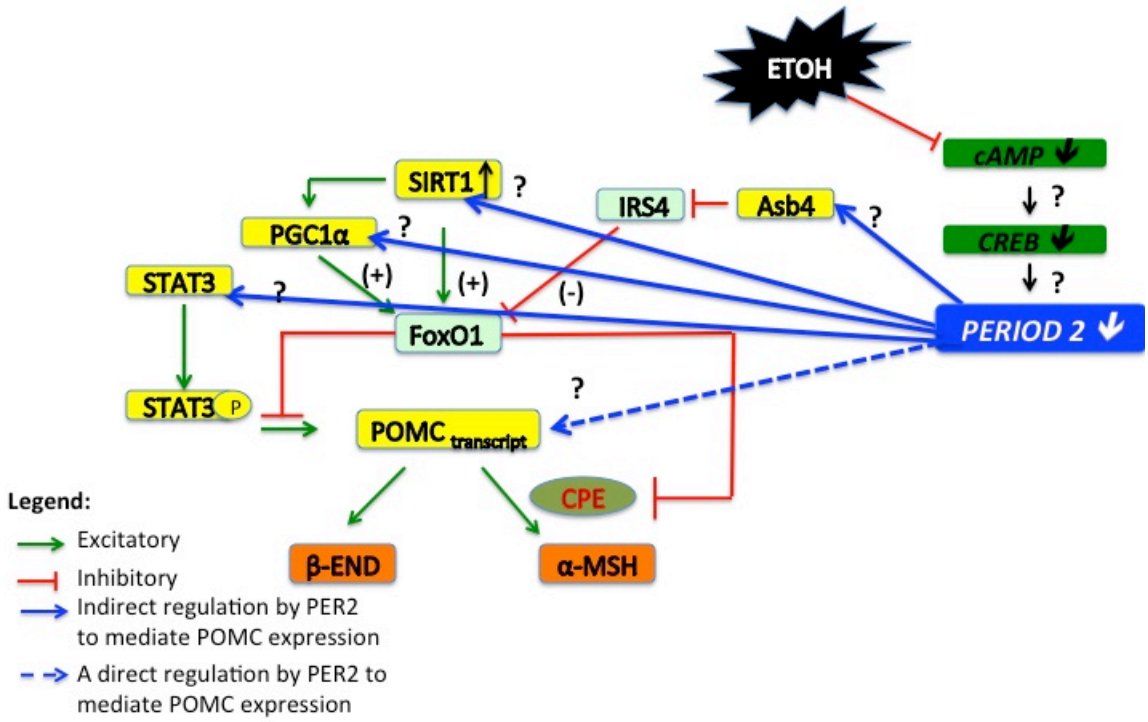


Figure 18. A proposed model for ethanol mechanism of action via PER2. cAMP-CREB pathways represent short routes connecting extracellular stimuli to the nucleus via the cytoplasm. Cytoplasmic proteins often display an on/off transmission switch, In this model ethanol may block the effect of PER2 regulation on the opioidergic system by disrupting the synthesis of cAMP upstream, resulting in reduction in CREB expression, leading to PER2 reduction. In this manner, the POMC neuronal function is altered.

PHYSIOLOGICAL EFFECT OF NEONATAL ALCOHOL EXPOSURE ON GLUCOSE LEVEL

It is believed that ethanol exposure declines any energy-based mechanism in the organism leading to neuronal death. This explains the findings outline in chapter 3 that demonstrate that ethanol inhibits and shuts down the rhythmicity of metabolic related genes, especially *Sirt1* and *PGC1α,* which function to stimulate gluconeogenesis. As a result, this effect can lead to loss of energy and/or glucose transport loss as suggested by other studies. Similarly, Yang's group demonstrated that Per2 deficient mice lack a recognizable corticosterone rhythm even though they can produce corticosterone normally in different forms of stress [423]. Moreover, Englund's lab had associated *Per2* 10870 polymorphism with reduced serum glucose levels [408]. It is unknown whether ethanol exposure on *Pomc*-expressing neurons has an impact on other peripheral metabolic systems. Since *Pomc*-expressing neurons modulate metabolic function, we propose that ethanol may hinder glucose homeostasis and this effect may be mediated through the PER2 gene.

Therefore, the glycemic circadian profile and glucose tolerance test were studied in control (AD and PF) and ethanol (AF) treated groups in C57BL/6 and Per2 mutant 90-day old male mice. The ethanol treated group from C57BL/6 and Per2 deficient mice PF group showed no significant difference. A similar observation was made with the Per2 mutant mice AD group with the exception of certain time points. All this information provides support to the concept that the Per2 gene mediates the alcohol effect on metabolic function of POMC.

Other possible implications of the hypoglycemic state in gestational ethanol treated animals:

The hypoglycemic state incurred during gestational ethanol treatments may be multifactorial in nature and outlined as follows: 1. The type of mice strain utilized: Mouse models produced through genetic modification have been generated in a variety of mouse strains. It is widely recognized that the background mouse strain can influence phenotypes. Several examples have been described where identical genetic mutations in different inbred mouse strains result in different phenotypes [424-426]. Therefore, we can presume that a genetic background issue may misperceive the data presented in the appendix section. However, C57BL/6 mice are a common background strain used to study glucose metabolism. A study by Berglund et al., demonstrated that C57BL/6 mice strain show an intermediate physiological response to glucose challenge in comparison to129X1/Sv, FVB/N, and DBA/2 mice [427]. Further, C57BL/6 mice displayed an in vivo physiological response to hyperinsulinemia at euglycemic and hypoglycemic state. These effects were intermediate relative to other strains. This finding is reassuring and suggests that C57BL/6 mice are suitable model for studies of glucose homeostasis.

2. The maintenance of systemic serum glucose levels depends on a combination of physiological processes including: intestinal absorption, glycogenolysis and gluconeogenesis. Ethanol induction of a hypoglycemic state requires a prolonged fasting state to exhaust the first two mechanisms (intestinal absorption and glycogenolysis) [428]. Moreover, ethanol also reduces gluconeogenesis by increasing NADH/NAD ratio, which has an adverse effect on conversion of substrates such as lactate and glutamate to pyruvate and α-ketoglutarate, which are necessary for gluconeogenesis [428]. In the

absence of gluconeogenesis, the liver contains enough glycogen to maintain normoglycemia for about 8 to 10 hours, thus as ethanol exposure inhibits gluconeogenesis, hypoglycemia may result very fast after glycogen stores are depleted [428, 429]. This may be the probable reasoning for the effect observed in our model system, and perhaps it is under these conditions that ethanol acts on the PER2 to mediate reduction of SIRT1 and PGC1α gene expression. Alternatively, under ethanol challenge, the ratio of NAD+/NADH ratio may be altered resulting in an increase of NADH, thus affecting SIRT1 induction.

Our supplemental data analysis shows that ethanol- treated groups exhibit a nadir in serum glucose levels between ZT23 to 11ZT (represented in LD time as 6 am to 2 pm). Similar data was publihed by Kosenko et al., 1986 who used a chronic ethanol model system, to postulate that ethanol treated groups exhibit an alcoholic hypoglycemic state between the hours of 10 am to 1 pm in comparison to control groups. Further, serum glucose in ethanol treated mice approached similar levels observed in control counterparts after 1 pm [430, 431]. However our data demonstrated that the serum glucose in the ethanol group increases after 1 pm but did not reach comparable levels to the control group. We postulate that postnatal ethanol treatment may hinder other mechanisms that are responsible for maintaining the glycemic rhythmicity and basal glycemic control.

In addition, our ethanol model system involved treating the mice pups for five days between PD2-PD7, after this treatment the animals were left alone until postnatal day ninety. The aforementioned study also showed that after 24 hr. of ethanol withdrawal serum glucose levels dropped significantly in the treated groups [430], which may

suggest that the hypoglycemic phenomena that is observed in our model system may be a result of an ethanol withdrawal effect that lasts until adulthood.

Moreover, Kosenko's paper explains that a liver/blood barrier for glucose forms during the 24h period after withdrawal of ethanol consumption, this barrier results in the prevention of transport of free glucose from the liver into the systemic circulation, resulting in severe hypoglycemia despite adequate stores of liver glycogen [431]. This may account for another probable explanation to the hypoglycemic state that is observed in our model system. Since in our model system ethanol exposure originates at the postnatal developmental phase, this alcohol effect may result in the formation of a liver/blood glucose barrier early in development that may be irreversible. As mentioned earlier both the metabolic and circadian signaling in the hypothalamus mature during the postnatal days, leading us to postulate that ethanol neonatal treatment hinders such metabolic cues affecting not only the *Pomc*-expressing neurons but also other metabolic processes.

3. Another possibility for the observed hypoglycemic effect exerted by the ethanol administration may be that the basal glycemic level in these ethanol treated animals may have an altered set point. It is known that a hypoglycemic state leads neurologic surplus (confusion, altered behavior, coma); with a rapid relief of such symptoms following administrations of exogenous carbohydrates. However our ethanol treated animals exhibited a normal behavior with a hypoglycemic state. Our glucose tolerance test data revealed an altered glucose load in ethanol-exposed animals in comparison to control groups. This suggests that the mechanisms that are responsible for the control of glycemic response to glucose challenges and the rhythmicity of serum glucose levels may

be hindered by ethanol exposure. Although this phenomenon is rare, Sood et al., demonstrate that this scenario is possible. Sood et al., revealed the possibility for an alteration in the basal glycemic set point that may result in a hypoglycemic state [432]. However one should note that a hypoglycemic state might arise due to other reasons such as enzyme deficiencies that affect carbohydrate metabolism. These deficiencies can interfere with the body's ability to process natural sugars, such as fructose and galactose, glycogen, or other metabolites; or due to hormonal deficiencies such as lack of pituitary or adrenal hormones. Since neonatal ethanol exposure may hinder metabolic signaling pathways, it may also adversely affect neuro- hormonal output.

Taken all these experimental and clinical studies into consideration we may speculate that ethanol exposure during development can dramatically alter important physiological processes that can eventually predispose FASD or FAS patients to metabolic disorders.

FINAL CONCLUSION:

It may be concluded that neonatal exposure to ethanol detrimentally alters the fetal programming of neuronal POMC function by changing both the circadian coordination and the metabolic signaling cue in these cells. We showed that the Per2 gene is necessary to maintain the ethanol response in *Pomc* neurons. We also showed that the Per2 gene mediates the expression and the response to ethanol of key metabolic genes in the MBH. To further analyze these findings, single Pomc expressing cells were isolated and examined at different time points to investigate neonatal alcohol and *Per*2 gene interaction. These studies identified that PER2 deficiency seizes the circadian

profile of key clock genes (*Bmal1, Per1 and Per2*) and metabolic genes (*Pomc*, *Stat3, Asb4, Sirt1*, and *Pgc1α*). Furthermore, alcohol treated groups of C57BL/6 mice showed an altered circadian profile and a reduction in the expression of these genes. Also, *Period*2 mutation prevented fetal alcohol effects on the circadian expression of POMC and metabolic genes. Our data suggest the possibility that PER2 may play the role of a metabolic timekeeper by influencing the circadian profiles of *(Pomc, Stat3, Asb4, Sirt1*, and *Pgc1α)*. However, more studies are needed to determine the mechanism by which PER2 governs *Pomc* gene expression.

The results provided in this thesis identified a novel role of a circadian clock gene Per2 in mediation of alcohol fetal programming on the stress and metabolic regulation. Clock genes are important cellular machineries governing not only circadian expression of cellular activities but have recently been shown to be key signaling elements governing the cell proliferation, endocrine functions, metabolic regulation and behaviors. Hence, future investigations on how Per2 mediates alcohol fetal programming on POMC cells may identify novel approaches to manage many behavioral and endocrine problems of FASD patients.

CHAPTER 6

SUPPLEMENTARY DATA

RATIONALE AND METHODS

The *Period* 2 gene (*Per*2) is notably involved in regulating the circadian clock. Recently, abnormalities in circadian clock machinery and of glucose homeostasis have been reported in offspring having been exposed to ethanol in utero or postnatal period. Our gene profile data has demonstrated that PER2 regulates the circadian rhythmicity of key genes important in maintaining metabolic function in the arcuate nucleus of the hypothalamus (chapter 3). Hence the question arose - whether *Per*2 gene may mediate postnatal alcohol effect on glycemic rhythm. Therefore, we investigated the effect of postnatal alcohol exposure on both circadian rhythm of serum glucose and glucose tolerance in 90 days old *Per2*^{Brdml} mutant and C57BL/6 male mice.

Animal Model

Pregnant C57BL/6 mice (Charles River) and *Per2*^{Brdml} (Jackson Laboratories, Bar Harbor, ME) mutant mice were individually housed in 12 hour light/12 hour dark cycles. The newborn mice (C57BL/6 and *Per2*^{Brdml}mutant mice) were treated with ethanol from postnatal day (PD)2-7. At day of treatment, two pups from each litter were fed by intubation with milk formula containing 11.34%(vol/vol) alcohol in milk formula (AF; 0.1-0.2 ml/animal; during a period of 1 minute) yielding a total daily ethanol dose of 2.5g/Kg; or an isocaloric volume of maltose dextrin (PF); or left alone (AD). The feeding was conducted at 1000 and 1200 h daily. After feeding, the pups were immediately returned to the litter. Pups then remained with their litter until postnatal day 22, when they were weaned, housed by treatment received, and given rodent chow meal and water

ad libitium. Males at 90 days old were used for this study. Animal care and treatment were performed in accordance with institutional guidelines and were approved by Rutgers Animal Care and facilities committee and complies with NIH policy.

Glucose Measurement & Analysis

At 90 days old, the 3 groups (AD, PF, and AF) of male subject mice (C57BL/6 and *Per2*^{Brdml}) were subjected to an 18-hour fasting period. Serum glucose levels were measured with 2.0 µl blood from a nicked tail vein using a Contour Blood Glucose Monitoring System (Bayer). Glucose measurements were obtained starting at 6pm (11 ZT) or 10 am (3 ZT), every 4 hours for a cycle of 24 hours.

Oral Glucose Tolerance Test

The glucose tolerance test was performed in three groups AD (ad libitum), PF (Pair-fed) and AF (PD2-PD7 alcohol fed) on mice fasted for 18 hours at 3 months of age. Mice were given a single dose orally (2g/kg body weight) of D-dextrose (Sigma) after a baseline glucose check. Circulating glucose levels were measured at indicated times after glucose exposure.

Statistical Analysis

Data are mean ± SEM of 6 animals per time point. One-way ANOVA with the Dunnett post-test were used to assess differences in glucose levels between time points of the same group. In the Dunnett post-test, the lowest value was chosen as the control. To assess differences between wild types and Per2 mutants, we performed two-way ANOVA with Bonferroni post-test. The values of glucose measurements between wild type and mutant mice were compared using student t test. A P value of less than 0.05 was considered significant

RESULTS

Postnatal ethanol effects on circadian rhythm of plasma glucose in Per2 mutant and wildtype mice

Previous data revealed that *Pomc* –expressing neurons from Per2 mutant mice showed no rhythmic profile of metabolic genes (*Pomc, Stat3, Asb4, Sirt1*, and *Pgc1α).* The majority of these genes play a role in mediating glucose homeostasis. Thus it was speculated that PER2 might mediate glucose homeostasis.

The data indicates that glucose levels in control groups (AD and PF) were rhythmic in C57BL6 mice. The highest blood glucose levels were observed during the dark phase between 15ZT to 3ZT, which are the times were the rodents are most active and are actively consuming food. The nadir points were observed between 7ZT and 11ZT, this is represented by the light phase. On the contrary, the data demonstrated that both AD and PF-treated $Per2^{Brdml}$ mutant mice exhibited a glucose rhythm shift that resulted in an earlier peak that initiated at 11 ZT as compared to the earlier peak observed at 15ZT in C57BL6 mice during the circadian cycle (Fig. 16 and 17). The basal glucose levels were not affected in the control groups (AD and PF) of $Per2^{Brdml}$ mutant mice as predicted before in chapter 3 (Figure 17).

The new data demonstrates that PER2 does not control blood glucose circadian profile. However, new evidence now reveals that PER2 may regulate glucose rhythm in part by serving as a timekeeper to maintain the resetting cue between the nadir and climax points.

The postnatal ethanol treatment resulted in a decline of glucose rhythm levels in C57BL6 mice during the adult period. Suggesting the ethanol mechanism of action is

hindering another mechanism that may be involved in maintaining basal glucose rhythmicity. Interestingly, postnatal ethanol treatment resulted in enhanced circadian blood glucose levels during climax times in Per2^{Brdml} mutant mice during the adult period (Fig. 16 and17). This phenomena is very intriguing, the fact that the shift peak is observed in all (AD, PF, and AF) treated groups in Per2 mutant mice demonstrates that PER2 may set the time when blood glucose levels reaches its climax (Fig. 17). However, the finding that the alcohol treated group exhibit an enhanced blood glucose level during the peak times suggest the possibility that perhaps ethanol mechanism of action is hindering another mechanism that are involved in establishing a basal glucose level set point (meaning sustaining the basal glucose level) as opposed to regulating the nadir and climax set time.

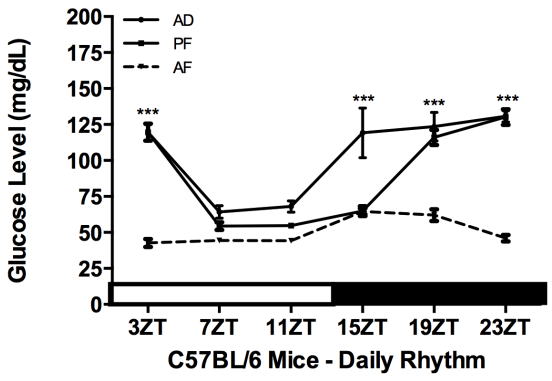


Figure. 19. Effect of postnatal ethanol exposure on circadian rhythm of blood glucose in C57BL/6 mice. ***, P <0.001, Alcohol-fed (AF) compared to Pair-fed (PF) and Ad libitum-fed (AD) males. N=6

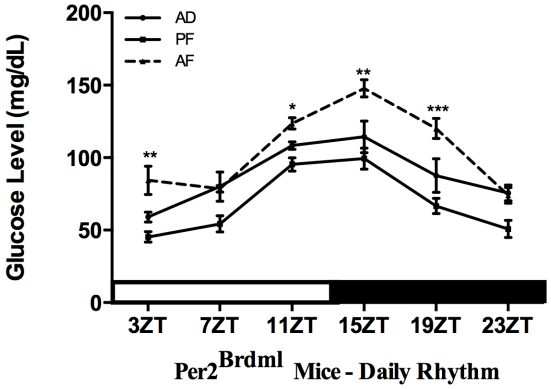


Figure. 20. Effect of postnatal ethanol exposure on circadian rhythm of blood glucose in *Per2^{Brdml}* mutant mice. , ***, p <0.001; **, p <0.01; *, p <0.05;Alcohol-

fed (AF) compared to Pair-fed (PF) and Ad libitum-fed (AD) males. N=6.

Postnatal ethanol effects on glucose tolerance test in per2 mutant and wildtype mice

A glucose tolerance test assesses the disposal of a glucose load administered via oral dosing. The results of a glucose tolerance test are determined by insulin secretion, insulin action, and glucose effectiveness . To determine the effect of ethanol on glucose tolerance, C57BL/6 and *Per2*^{Brdml} mutant mice were fasted for 18 h, and an oral glucose tolerance test was performed using 2 g/kg glucose (Figure 18 and 19).

The results demonstrated that control groups (AD and PF) in C57BL/6 mice exhibited a normal glucose tolerant test determined by the glucose curve that shows a glucose peak at ~ 350 mg/dL in 15 minutes and it subsequently recedes to normal glucose levels of ~ 160 mg/dL after two hours (Figure 3). However in the ethanol treated group, an altered glucose curve was observed that resulted in a median peak at ~ 230 mg/dL in 15 minutes and this peak immediately receded to ~ 120 mg/dL after two hours. The significant difference observed between control groups (AD and PF) and ethanol group were between 15 – 60 minutes. This data revealed that the ethanol treated group has an enhanced glucose load clearance than control groups. Thus, ethanol mechanism of action may target both the insulin secretion and insulin action pathways to deliver such effect.

However, the *Per2*^{Brdml} mutant mice displayed no significant difference in the glucose tolerant curves in all three groups (AD, PF and AF) (Figure 19). The glucose levels rendered in *Per2*^{Brdml} mutant mice did not differ from C57BL/6 mice. This data

suggest that PER2 may mediate the glucose tolerance response to ethanol. More evidence needs to be elucidated to support this proposal.

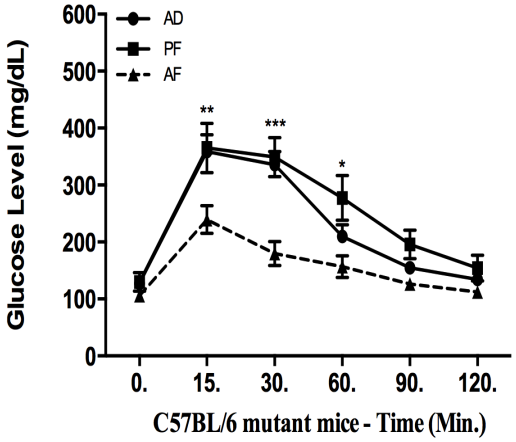


Figure. 21. Effect of postnatal ethanol exposure on glucose tolerance test in C57BL/6 mice. ***, p <0.001; **, p < 0.01; *, p < 0.05, Alcohol-fed (AF) compared to Pair-fed (PF) and Ad libitum-fed (AD) males. N=6

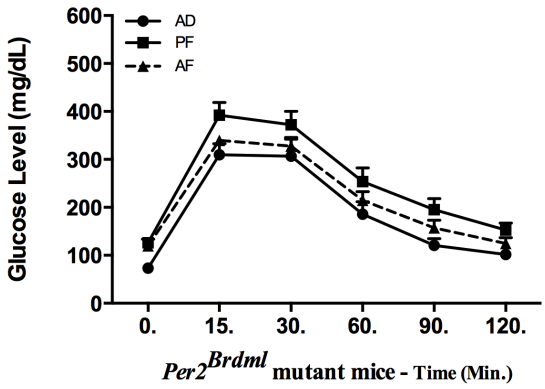


Figure. 22. Effect of postnatal ethanol exposure on glucose tolerance test in *Per2*^{Brdml} mutant mice. , ***, P <0.001; **, P <0.01; *, P <0.05; Alcohol-fed (AF) compared to Pair-fed (PF) and Ad libitum-fed (AD) males. N=6

APPENDIX

Most of the information obtained from the studies comprising this Dissertation has been published recently in the following journals:

Agapito MA, JC Barreira, Zhang C, DK Sarkar. Fetal alcohol exposure disrupts metabolic signaling in hypothalamic proopiomelanocortin neurons via a circadian mechanism. 2013. Alcohol Clinical and Experimental Research (in press).

Agapito MA, Barreira JC, Logan RW, Sarkar DK. Evidence for Possible Period 2 Gene Mediation of the Effects of Alcohol Exposure During the Postnatal Period on Genes Associated with Maintaining Metabolic Signaling in the Mouse Hypothalamus. 2012. Alcohol Clin Exp Res. (Epub ahead of print)

Agapito MA, Mian N, Boyadjieva NI, Sarkar DK. Period 2 gene deletion abolishes beta-endorphin neuronal response to ethanol. 2010. Alcohol Clin Exp Res. 34(9): 1613-8.

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