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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

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Background: Animals exposed to alcohol during the developmental period develop circadian disturbances and metabolic problems that often persist during their adult period. In order to study whether alcohol and the circadian clock interact to alter metabolic signaling in the hypothalamus, we determined whether postnatal alcohol feeding in mice permanently alters metabolic sensing in the hypothalamus. Furthermore, we evaluated whether the effect of circadian disruption via *Period* 2 (*Per*2) gene mutation prevents alcohol's effects on metabolic signaling in the hypothalamus.

Methods: *Per*2 mutant and wild-type male and female mice of the same genetic background were given a milk formula containing ethanol (EtOH; 11.34% vol/vol) from postnatal day (PD) 2 to 7 and used for gene expression and peptide level determinations in the hypothalamus at PD7 and PD90.

Results: We report here that postnatal alcohol feeding reduces the expression of proopiomelanocortin (*Pomc*) gene and production of β-endorphin and α-melanocyte stimulating hormone (α-MSH) in the hypothalamus that persists into adulthood. In addition, expressions of metabolic sensing genes in the hypothalamus were also reduced as a consequence of postnatal alcohol exposure. These effects were not sex-specific and were observed in both males and females. Mice carrying a mutation of the *Per*2 gene did not show any reductions in hypothalamic levels of *Pomc* and metabolic genes and β-endorphin and α-MSH peptides following alcohol exposure.

Conclusions: These data suggest that early-life exposure to alcohol alters metabolic sensing to the hypothalamus possibly via regulating *Per*2 gene and/or the cellular circadian clock mechanism.

Key Words: Circadian Rhythm, Fetal Alcohol Spectrum Disorders, Metabolic Disorders, POMC Neurons.

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 (Hellemans et al., 2010; Schneider et al., 2011), altered metabolic functioning (Chen and Nyomba, 2003), impairment in the immune response (Arjona et al., 2006), to disruptions in circadian rhythms (Chen et al., 2006; Handa et al., 2007). A critical component for the regulation of stress, metabolic, and immune functions is the proopiomelanocortin (*Pomc*) gene (Boyadjieva et al., 2009; Sarkar et al., 2008), which has been shown to be a target of alcohol and clock genes (Agapito et al., 2010; Chen et al., 2006). 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 (Appleyard et al., 2003; Coll et al., 2000; Millington, 2007), and immune regulation (Boyadjieva et al., 2006). *Pomc* gene expression abnormalities are associated with obesity, hyperphagia, diabetes (Baker et al., 2005; Chen et al., 2005; Mizuno et al., 2003), and cancer (Sarkar et al., 2008). 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

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(*Pgc*1α), are associated with *Pomc* function and play regulatory roles in metabolism. For example, *Stat*3 regulates *Pomc* gene expression (Xu et al., 2007), and the mutation of this gene is associated with a severe obesity phenotype (Gao et al., 2004). *Sirt*1 is another key metabolic signaling gene that orchestrates adaptation to changing metabolic states in peripheral tissues (Cohen et al., 2004). Recently, *Sirt*1 has been found to localize in the arcuate nucleus of the hypothalamus and colocalizes with *Pomc*-producing neurons (Ramadori et al., 2008). In the liver, both *Sirt*1 and *Pgc*1α peptides play a role to mediate the NAD+ mechanism. Interestingly, *Pgc*1α also colocalizes 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 (Lieber et al., 2008). Little is known about the function of *Asb*4, 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 (Li et al., 2007), 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 (Chen et al., 2006; Grimaldi et al., 2009). Recently, clock genes have been connected to formation and progression of many diseases related to metabolic disorders (Ando et al., 2009; Bass and Takahashi, 2010). Clock genes, such as negative regulators, *Period* (*Per*1,2,3), and *Cryptochrome* (*Cry*1,2), 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 (Ko and Takahashi, 2006). 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 (Arjona et al., 2006; Chen et al., 2006). Additionally, *Per*2 gene mutant ($mPer2^{Brdm1}$) mice display enhanced alcohol consumption and preference (Spanagel et al., 2005), whereas $Per1^{Brdm1}$ mutant mice do not show such an enhancement in alcohol drinking behavior (Zghoul et al., 2007). Evidence has shown alcoholics with a specific set of polymorphisms in the *Per*2 gene consume less alcohol than alcoholics without the polymorphisms (Spanagel et al., 2005). Furthermore, under a metabolic disease state, the expression of *Per* genes is altered in peripheral tissues (Garaulet et al., 2010). 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 (Zheng et al., 1999), 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 Agapito et al., 2010). 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 (Blizard et al., 2004). 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 (Church and Feller, 1979). These data support that the tyrosinase deficiency will have very little consequences in alcohol–response studies. $Per2^{Brdm1}$ and C57BL/6 mice models have also been used previously in determining the role of *Per*2 in alcohol effects on the brain (Agapito et al., 2010; Perreau-Lenz et al., 2009).

*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 $mPer2^{Brdm1}$ 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 $mPer2^{Brdm1}$ 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 (Sarkar et al., 2007), 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 (Agapito et al., 2010; Chen et al., 2006) 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 (Cheung and Hammer, 1995; Sarkar and Minami, 1995), 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 (Bohler et al., 1991). 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 (Handa and Rodriguez, 1991; Lino et al., 1993). Both 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 animal protocol used was approved by the Rutgers Animal Care and Facilities Committee.

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.

RESULTS

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. 1*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. 1*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. 1*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. 1*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. 1*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. 1*D*1–*D*3).

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,

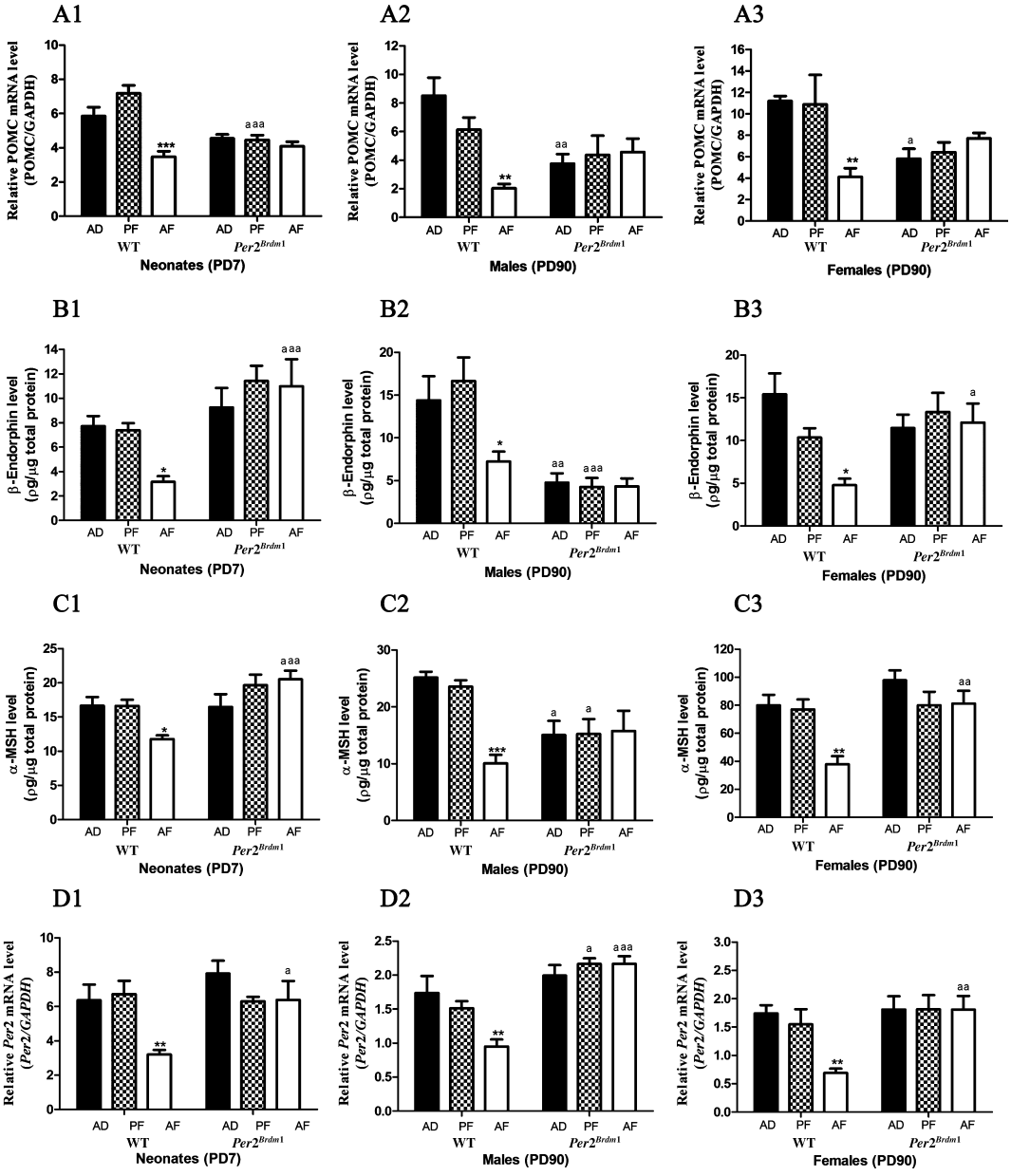

Fig. 1. Effect of postnatal ethanol exposure on levels of proopiomelanocortin (*Pomc*) gene mRNA (**A**), β-endorphin (**B**), α-melanocyte stimulating hormone (α-MSH) (**C**), and *Per2* (**D**) in the mediobasal hypothalamus (MBH) at PD7 and PD90 in C57BL/6 and $Per2^{Brdm1}$ mice. Pups fed with milk 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 *mPer2* mutant mice in each feeding group ($^{a}p < 0.05$, $^{aa}p < 0.01$, $^{aaa}p < 0.001$).

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. 2). 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.

DISCUSSION

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

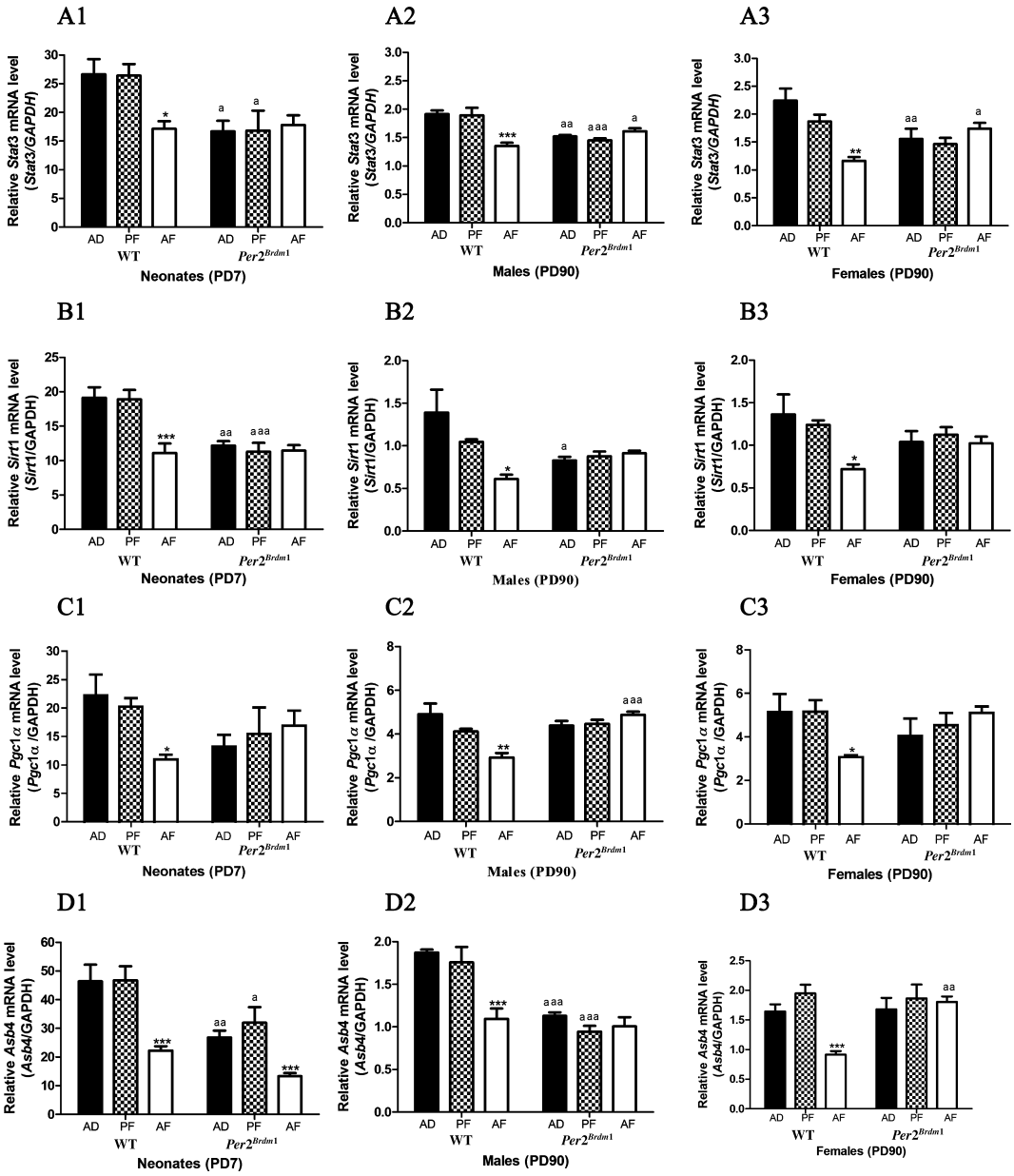

Fig. 2. Effect of postnatal ethanol exposure on mRNA levels of *Stat*3 (**A**), *Sirt*1 (**B**), *Pgc*1α (**C**), and *Asb*4 (**D**) in the mediobasal hypothalamus (MBH) at PD7 and PD90 in C57BL/6 and *Per2*^{Brdm1} mice. Pups were fed with a milk formula containing alcohol (AF), pair-fed isocaloric 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 *mPer2* mutant mice in each feeding group ($^{a}p < 0.05$, $^{aa}p < 0.01$, $^{aaa}p < 0.001$).

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 *mPer2* mutant mice. Prenatal EtOH or *Per2* 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 *Per2* mutation are in general similar on *Pomc* and metabolic sensing genes, and because *Per2* mutation prevents EtOH to further alter metabolic gene expression, our data strongly suggest that *Per2* may mediate EtOH's action on metabolic sensing genes.

Our findings on the involvement of *Per2* gene in mediating EtOH's action on POMC neurons to alcohol are consistent with several previous indirect evidences. For example, Agapito and colleagues (2010) have demonstrated that *Per2* mutation prevented β-endorphin stimulatory and inhibitory responses to acute and chronic EtOH challenges in a cell culture system. Also, prenatal EtOH decreases *Per2* mRNA levels in the arcuate

nucleus where many *Pomc* neuronal cells are localized (Chen et al., 2006). Additionally, *Per*2 gene is identified in laser captured microdissected β-endorphin neurons (Chen et al., 2006), indicating that POMC-producing neurons express the *Per*2 gene. Also, a population of POMC neurons produces and releases glutamate (Hentges et al., 2009), which is also a target of *Per*2 mutation (Spanagel et al., 2005).

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 (Hirayama et al., 2007). However, it is clear, at least in *Drosophila*, that PER is associated with DNA (Yoshitane et al., 2009). Moreover, recent studies in rat pituitary GH3 cells have shown PER proteins acting directly on the promoter of pituitary prolactin (Bose and Boockfor, 2010). 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 (Ervin, 2009). There is also a report that some fetal alcohol children show abnormal oral glucose tolerance tests with increased plasma insulin response (Castells et al., 1981). 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 (Di Lorenzo et al., 2003; Turek et al., 2005). A number of studies have also connected the clock genes with metabolic sensing in the hypothalamus (reviewed in Gatfield and Schibler, 2008). 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 (Di Lorenzo et al., 2003). 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 (Laposky et al., 2006). Furthermore, FASD patients are known to have altered sleep patterning (Burd and Wilson, 2004; Jan et al., 2010). 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 EtOH-influenced expression of certain metabolic sensing genes (*Stat*3, *Sirt*1, *Pgc*1α, and *Asb*4) is regulated by *Per*2. All these metabolic genes were found to be expressed 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.

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REFERENCES

Agapito M, Mian N, Boyadjieva NI, Sarkar DK (2010) Period 2 gene deletion abolishes beta endorphin neuronal response to ethanol. Alcohol Clin Exp Res 34:1613–1618.

Ando H, Takamura T, Matsuzawa-Nagata N, Shima KR, Eto T, Misu H, Shiramoto M, Tsuru T, Irie S, Fujimura A, Kaneko S (2009) Clock gene expression in peripheral leucocytes of patients with type 2 diabetes. Diabetologia 52:329–335.

Appleyard SM, Hayward M, Young JI, Butler AA, Cone RD, Rubinstein M, Low MJ (2003) A role for the endogenous opioid beta-endorphin in energy homeostasis. Endocrinology 144:1753–1760.

Arjona A, Boyadjieva N, Kuhn P, Sarkar DK (2006) Fetal ethanol exposure disrupts the daily rhythms of splenic granzyme B, IFN-gamma, and NK cell cytotoxicity in adulthood. Alcohol Clin Exp Res 30:1039–1044.

Baker M, Gaukrodger N, Mayosi BM, Imrie H, Farrall M, Watkins H, Connell JMC, Avery PJ, Keavney B (2005) Association between common polymorphisms of the proopiomelanocortin gene and body fat distribution. Diabetes 54:2492–2496.

Bass J, Takahashi JS (2010) Circadian integration of metabolism and energetics. Science 330:1349–1354.

Blizard DA, Vandenbergh DJ, Jefferson AL, Chatlos CD, Vogler GP, McClearn GE (2004) Effects of periadolescent ethanol exposure on alcohol preference in two BALB substrains. Alcohol 34:177–185.

Bohler HC Jr, Tracer H, Merriam GR, Petersen SL (1991) Changes in proopiomelanocortin messenger ribonucleic acid levels in the rostral periarcuate region of the female rat during the estrous cycle. Endocrinology 128:1265–1269.

Bose S, Boockfor FR (2010) Episodes of prolactin gene expression in GH3 cells are dependent on selective promoter binding of multiple circadian elements. Endocrinology 151:2287–2296.

Boyadjieva N, Advis JP, Sarkar DK (2006) Role of beta-endorphin, corticotropin-releasing hormone and autonomic nervous system in mediation of the effect of chronic ethanol on natural killer cell cytolytic activity. Alcohol Clin Exp Res 30:1761–1767.

Boyadjieva NI, Ortigüela M, Arjona A, Cheng X, Sarkar DK (2009) Beta-endorphin neuronal cell transplant reduces corticotropin releasing hormone hyperresponse to lipopolysaccharide and eliminates natural killer cell functional deficiencies in fetal alcohol exposed rats. Alcohol Clin Exp Res 33:931–937.

Burd L, Wilson H (2004) Fetal, infant, and child mortality in a context of alcohol use. Am J Med Genet C Semin Med Genet 127C:51–58.

Castells S, Mark E, Abaci F, Schwartz E (1981) Growth retardation in fetal alcohol syndrome. Unresponsiveness to growth-promoting hormones. Dev Pharmacol Ther 3:232–241.

Chen CP, Kuhn P, Advis JP, Sarkar DK (2006) Prenatal ethanol exposure alters the expression of period genes governing the circadian function of beta-endorphin neurons in the hypothalamus. J Neurochem 97:1026–1033.

Chen L, Nyomba BL (2003) Effects of prenatal alcohol exposure on glucose tolerance in the rat offspring. Metabolism 52:454–462.

Chen Y, Snieder H, Wang X, Kaviya B, Mc Caffrey C, Spector TD, Carter ND, O' Dell SD (2005) Proopiomelanocortin gene variants are associated with serum leptin and body fat in a normal female population. Eur J Hum Genet 13:772–780.

Cheung S, Hammer RP Jr (1995) Gonadal steroid hormone regulation of proopiomelanocortin gene expression in arcuate neurons that innervate the medial preoptic area of the rat. Neuroendocrinology 62:283–292.

Church AC, Feller D (1979) The influence of mouse genotype on the changes in brain cyclic nucleotide levels induced by acute alcohol administration. Pharmacol Biochem Behav 10:335–338.

Cohen HY, Miller C, Bitterman KJ, Wall NR, Hekking B, Kessler B, Howitz KT, Gorospe M, de Cabo R, Sinclair DA (2004) Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase. Science 305:390–392.

Coll AP, Farooqi IS, Challis BG, Yeo GS, O'Rahilly S (2000) Proopiomelanocortin and energy balance: insights from human and murine genetics. J Clin Endocrinol Metab 89:2557–2562.

Di Lorenzo L, De Pergola G, Zocchetti C, L'Abbate N, Basso A, Pannacciulli N, Cignarelli M, Giorgino R, Soleo L (2003) Effect of shift work on body mass index: results of a study performed in 319 glucose-tolerant men working in a Southern Italian industry. Int J Obes Relat Metab Disord 27:1353–1358.

Ervin BR (2009) Prevalence of Metabolic Syndrome Among Adults 20 Years of Age and Over, by Sex, Age, Race and Ethnicity, and Body Mass Index: United States, 2003–2006. Natl Health Stat Rep 13:1–8.

Gao Q, Wolfgang MJ, Neschen S, Morino K, Horvath TL, Shulman GI, Fu XY (2004) Disruption of neural signal transducer and activator of transcription 3 causes obesity, diabetes, infertility, and thermal dysregulation. Proc Natl Acad Sci USA 101:4661–4666.

Garaulet M, Corbalán-Tutau MD, Madrid JA, Baraza JC, Parnell LD, Lee YC, Ordovas JM (2010) PERIOD2 variants are associated with abdominal obesity, psycho-behavioral factors, and attrition in the dietary treatment of obesity. J Am Diet Assoc 110:917–921.

Gatfield D, Schibler U (2008) Circadian glucose homeostasis requires compensatory interference between brain and liver clocks. Proc Natl Acad Sci USA 105:14753–14754.

Grimaldi B, Nakahata Y, Kaluzova M, Masubuchi S, Sassone-Corsi P (2009) Chromatin remodeling, metabolism and circadian clocks: the interplay of CLOCK and SIRT1. Int J Biochem Cell Biol 41:81–86.

Handa RJ, Rodriguez EW (1991) A characterization of estrogen's influence on anterior pituitary androgen receptor: effect of bromocriptine treatment. Neuroendocrinology 53:12–19.

Handa RJ, Zuloaga DG, McGivern RF (2007) Prenatal ethanol exposure alters core body temperature and corticosterone rhythms in adult male rats. Alcohol 41:567–575.

Hellemans KG, Sliwowska JH, Verma P, Weinberg J (2010) Prenatal alcohol exposure: fetal programming and later life vulnerability to stress, depression and anxiety disorders. Neurosci Biobehav Rev 34:791–807.

Hentges ST, Otero-Corchon V, Pennock RL, King CM, Low MJ (2009) Proopiomelanocortin expression in both GABA and glutamate neurons. J Neurosci 29:13684–13690.

Hirayama J, Sahar S, Grimaldi B, Tamaru T, Takamatsu K, Nakahata Y, Sassone-Corsi P (2007) CLOCK-mediated acetylation of BMAL1 controls circadian function. Nature 450:1086–1090.

Jan JE, Asante KO, Conry JL, Fast DK, Bax MC, Ipsiroglu OS, Bredberg E, Loock CA, Wasdell MB (2010) Sleep health issues for children with FASD: clinical considerations. Int J Pediatr pii:639048.

Ko CH, Takahashi JS (2006) Molecular components of the mammalian circadian clock. Hum Mol Genet 15:R271–R277.

Laposky AD, Shelton J, Bass J, Dugovic C, Perrino N, Turek FW (2006) Altered sleep regulation in leptin-deficient mice. Am J Physiol Regul Integr Comp Physiol 290:R894–R903.

Li JY, Chai BX, Zhang W, Liu YQ, Ammori JB, Mulholland MW (2007) Ankyrin repeat and SOCS box containing protein 4 (Asb-4) interacts with GPS1(CSN1) and inhibits c-Jun NH2-terminal kinase activity. Cell Signal 19:1185–1192.

Lieber CS, Leo MA, Wang X, Decarli LM (2008) Effect of chronic alcohol consumption on Hepatic SIRT1 and PGC-1alpha in rats. Biochem Biophys Res Commun 370:44–48.

Lino Y, Takai Y, Ando T, Sugamata N, Maemura M, Takeo T, Ohwada S, Morishita Y (1993) Effect of toremifene on the growth, hormone receptors and insulin-like growth factor-1 of hormone-dependent MCF-7 tumors in athymic mice. Cancer Chemother Pharmacol 32:353–358.

Millington GW (2007) The role of proopiomelanocortin (POMC) neurons in feeding behavior. Nutr Metab (Lond) 4:18.

Mizuno TM, Kelley KA, Pasinetti GM, Roberts JL, Mobbs CV (2003) Transgenic neuronal expression of proopiomelanocortin attenuates hyperphagic response to fasting and reverses metabolic impairments in leptin-deficient obese mice. Diabetes 52:2675–2683.

Perreau-Lenz S, Zghoul T, de Fonseca FR, Spanagel R, Bilbao A (2009) Circadian regulation of central ethanol sensitivity by the mPer2 gene. Addict Biol 14:253–259.

Ramadori G, Lee CE, Bookout AL, Lee S, Williams KW, Anderson J, Elmquist JK, Coppari R (2008) Brain SIRT1: anatomical distribution and regulation by energy availability. J Neurosci 28:9989–9996.

Sarkar DK, Boyadjieva NI, Chen CP, Ortigüela M, Reuhl K, Clement EM, Kuhn P, Marano J (2008) Cyclic adenosine monophosphate differentiated beta-endorphin neurons promote immune function and prevent prostate cancer growth. Proc Natl Acad Sci USA 105:9105–9110.

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

Sarkar DK, Minami S (1995) Diurnal variation in luteinizing hormone-releasing hormone and beta-endorphin release in pituitary portal plasma during the rat estrous cycle. Biol Reprod 53:38–45.

Schneider ML, Moore CF, Adkins MM (2011) The effects of prenatal alcohol exposure on behavior: rodent and primate studies. Neuropsychol Rev 21:186–203.

Spanagel R, Pendyala G, Abarca C, Zghoul T, Sanchis-Segura C, Magnone MC, Lascorz J, Depner M, Holzberg D, Soyka M, Schreiber S, Matsuda F, Lathrop M, Schumann G, Albrecht U (2005) The clock gene Per2 influences the glutamatergic system and modulates alcohol consumption. Nat Med 11:35–42.

Turek FW, Joshu C, Kohsaka A, Lin E, Ivanova G, McDearmon E, Laposky A, Losee-Olson S, Easton A, Jensen DR, Eckel RH, Takahashi JS, Bass J (2005) Obesity and metabolic syndrome in circadian Clock mutant mice. Science 308:1043–1045.

Xu AW, Ste-Marie L, Kaelin CB, Barsh GS (2007) Inactivation of signal transducer and activator of transcription 3 in proopiomelanocortin (Pomc) neurons causes decreased pomc expression, mild obesity, and defects in compensatory refeeding. Endocrinology 148:72–80.

Yoshitane H, Takao T, Satomi Y, Du NH, Okano T, Fukada Y (2009) Roles of CLOCK phosphorylation in suppression of E-box-dependent transcription. Mol Cell Biol 29:3675–3686.

Zghoul T, Abarca C, Sanchis-Segura C, Albrecht U, Schumann G, Spanagel R (2007) Ethanol self-administration and reinstatement of ethanol-seeking behavior in Per1(*Brdm*1) mutant mice. Psychopharmacology 190:13–19.

Zheng B, Larkin DW, Albrecht U, Sun ZS, Sage M, Eichele G, Lee CC, Bradley A (1999) The mPer2 gene encodes a functional component of the mammalian circadian clock. Nature 400:169–173.