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Evidence for possible *Period2* gene mediation of the effects of alcohol exposure during the postnatal period on genes associated with maintaining metabolic signaling in the mouse hypothalamus

Maria A. Agapito, Jacklin C. Barreira, Ryan W. Logan, and Dipak K. Sarkar*

Endocrine Program, Department of Animal Sciences, Graduate Program of Neuroscience, Rutgers, The State University of New Jersey, New Brunswick, New Jersey, USA

Abstract

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 *Period2* (*Per2*) gene mutation prevents alcohol's effects on metabolic signaling in the hypothalamus.

Methods—*Per2* mutant and wild type male and female mice of the same genetic background were given a milk formula containing ethanol (11.34%; vol/vol) from postnatal day (PD)2–7 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 *Per2* 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 *Per2* gene and/or the cellular circadian clock mechanism.

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 et al., 2003), impairment in the immune response (Arjona et al., 2006), and disruptions in circadian rhythms (Chen et al., 2006; Handa et al., 2007). A critical component for regulation of stress, metabolic and immune functions is the proopiomelanocortin gene (*POMC*) (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

*Corresponding author address: Dr. Dipak K. Sarkar, Professor II and Director, Program of Endocrinology, The State University of New Jersey, Endocrine Research Facility, 67 Poultry Farm Lane, New Brunswick, NJ 08901, USA. Phone: 732-932-1529; Fax: 732-932-4134; sarkar@aesop.rutgers.edu.

responses due to developmental alcohol exposure may be in part due to 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 (ACTH) and α -, β , and γ -melanocyte stimulating hormones (MSH), which are involved in regulation of food intake, metabolism, stress response (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 (*Stat3*), ankyrin repeat and suppressor of cytokine signaling (SOCS) box-containing 4 (*Asb4*), sirtuin 1 (*Sirt1*), and peroxisome proliferator-activated receptor gamma coactivator1 α (*Pgc1 α*), are associated with POMC function and play regulatory roles in metabolism. For example, *Stat3* 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). *Sirt1* is another key metabolic signaling gene that orchestrates adaptation to changing metabolic states in peripheral tissues (Cohen et al., 2004). Recently, *Sirt1* 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 *Sirt1* and *Pgc1 α* peptides play a role to mediate the NAD⁺ mechanism. Interestingly, *Pgc1 α* also colocalizes in POMC neurons, however the role that it plays in these neurons is unknown. Furthermore, gene expression levels of *Sirt1* and *Pgc1 α* are altered by alcohol exposure in the livers of adult rats (Lieber et al., 2008). Little is known about the function of *Asb4*, however, recent literature has indicated that *Asb4* 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 *Asb4* (Li et al., 2007), thus indicating its importance in metabolic sensing in the brain.

Metabolic genes, in particular POMC and *Sirt1*, 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 et al., 2010). Clock genes, such as negative regulators, *Period* (*Per1,2,3*), and *Cryptochrome* (*Cry1,2*), and positive regulators, *Clock* and *Bmal1*, act in two tightly coupled transcriptional and translational feedback loops that are able to self-sustain a circadian rhythm (Ko and Takahashi, 2006). Interestingly, it appears that *Per2* 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, *Per2* gene mutant (*Per2^{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 *Per2* 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 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

Per2 mutant (*mPer2^{Brdml}*) 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^{Brdml}* mice carry a mutant *mPer2* 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 non-functional PER2 protein. Although the *Per2^{Brdml}* mutant mice have an albino phenotype, because it was engineered with a tyrosinase gene deficiency that affects the ability to make melanin, the *Per2^{Brdml}* 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 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 (Blizard et al., 2004). Furthermore, these two mouse strains (C57BL/6By and BALB/cByJ) showed no differences in their hypothermic response or the brain level of cGMP to the same ethanol dose (Church and Feller, 1979). These data support that the tyrosinase deficiency will have very little consequences in alcohol-response studies. *Per2^{Brdml}* and C57BL/6 mice models have also been used previously in determining the role of *Per2* in alcohol effects on the brain (Agapito et al., 2010; Perreau-Lenz et al., 2009).

Per2 mutant and wildtype 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. with *ad libitum* food and water. The *mPer2^{Brdml}* mutant mice were routinely genotyped to verify the *Per2* gene mutation. There were four primers used for detecting the *Per2* gene wild type and mutant variants. For the wildtype primer; forward-ctgggtggagaggctattc, reverse-aggtgagatgacaggagatc. For the mutant primer; forward-cattgggaggcacaagtcag and reverse-gagctcggaacacatctca.

Animal treatments

C57BL/6 pups and *Per2^{Brdml}* mutant pups (both sexes) were fed by intubation with milk formula containing either alcohol (alcohol-fed; AD) 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 ethanol (11.34%; vol/vol; 0.1–0.2 ml/animal; during a period of 1 minute). The feeding was conducted at 1000 and 1200 h from PD2–PD7. After feeding, the pups were immediately returned to the litter. Some of these animals were sacrificed one hour after the last feeding (1300 h) 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 ethanol 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 day of age. Female rats were ovariectomized bilaterally and subcutaneously implanted with a 0.5 cm estradiol-17 β -filled silastic capsule (Dow Corning Corp., Midland, MI) under pentobarbital anesthesia. In order 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 75–100 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 sacrificed at day 90 at 10 p.m., 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.

B-endorphin immunoassays and protein measurement

The level of β -endorphin in the MBH tissue was measured by enzyme immunoassay (EIA) 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 (Invitrogen, CA). Then the high-capacity cDNA reverse transcription kit from applied Biosystems (Foster City, CA) was used for the RT reaction. The complementary DNA was subjected to the real-time polymerase chain reaction (RT-PCR) on an ABI Prism 7500 sequence detector (Applied Biosystems, Foster City, CA). The Taqman probe primers (*GAPDH*, *POMC*, *Per2*, *Stat3*, *Asb4*, *Sirt1*, and *Pgc1 α*) were acquired from Applied Biosystems (Foster City, CA).

Statistics

Data are presented as mean $\pm$ SEM. The treatment effects and strain effects were determined by two-way ANOVA with post hoc analysis using the Bonferroni post-test. $P < 0.05$ was considered significant.

RESULTS

Comparison of the effects of ethanol on POMC neurons in control and *Per2* 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 *Per2* mutant mice following administration of alcohol via milk formula or control treatments for five days. As shown in Fig. 1A1–3, *POMC* mRNA levels in PF and AD mice show similar levels at PD7 and PD90 in both C57BL/6 and *Per2* 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 *Per2* mutant mice. Mean levels of *POMC* mRNA in control-treated C57BL/6 mice, in general, were higher than those in *Per2* 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. 1B1–3). 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 *Per2* 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 two genotypes, it was observed that adult control treated (AD and PF) C57BL/6 males had significantly higher levels of the

peptide than those in *Per2* mutants, while AF treated PD7 and PD90 C57BL/6 females had significant lower levels of β -endorphin than those in *Per2* mutant mice (Fig. 1B2),

The postnatal effects of ethanol on α -MSH levels in the MBH at PD7 and PD90 in C57BL/6 and *Per2* mutant are shown in Fig. 1C1–3. The MBH tissue levels of α -MSH in AD and PF groups were similar at all time points in both C57BL/6 and *Per2* 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 *Per2* mutant mice. When the endogenous levels of MBH α -MSH were analyzed between wild type and *Per2* mutant mice, it was observed that, like the effect seen for β -endorphin, α -MSH levels were significantly different in adult control treated males (*Per2* mutants had lower levels of protein) and AF treated PD7 and PD90 females (*Per2* mutants had higher levels of protein).

The effect of postnatal ethanol treatment on mRNA levels of *Per2* gene in the MBH tissues was also examined at PD7 and PD90 in both C57BL/6 and *Per2* mutant mice. Postnatal alcohol feeding suppressed *Per2* mRNA levels during the postnatal period that persisted during the adult period in both male and female in C57BL/6 mice (Fig. 1D1–3). Basal expression of *Per2* mRNA did not differ at any developmental phase nor did it show any dimorphic effect when compared to wild type mice (Fig. 1D1–3).

Comparison of the effects of prenatal ethanol on the expression of metabolic sensing genes in the MBH of control and *Per2* mutant mice

In order to test the role of *Per2* in ethanol's action on the metabolic sensing of POMC neurons, we compared the effects of postnatal exposure to ethanol on mRNA levels of *Stat3*, *Sirt1*, *Pgc1- α* and *Asb4* in MBH tissues of C57BL/6 and *Per2* mutant mice at PD7 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 *Per2* 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 *Asb4* mRNA levels on PD7 only. Genotype comparison revealed that on the postnatal phase on PD7, the mRNA levels of *Stat3*, *Sirt1* and *Asb4* in AD and PF groups showed a reduction in *Per2* mutant mice, compared to those in wild type mice (Fig. 2A–D). On PD90, AD and PF females showed no difference in the expression of all but the *Stat3* gene between C57BL/6 and *Per2* mutant mice, while AD and PF males showed a reduction in *Stat3* and *Asb4* mRNA levels, and AD males showed a reduction in *Sirt1* mRNA levels in *Per2* 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 ethanol treatment also reduces the expression of one of the circadian clock genes *Per2* that persists into the adulthood. Interestingly, in mice carrying a mutation of the *Per2* 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 *Per2* mutant mice. Prenatal ethanol 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 ethanol and *Per2* mutation are in general similar on *POMC* and metabolic sensing genes, and because *Per2* mutation prevents ethanol to further alter

metabolic gene expression, our data strongly suggest that *Per2* may mediate ethanol's action on metabolic sensing genes.

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 *Per2* mutation prevented β -endorphin stimulatory and inhibitory responses to acute and chronic ethanol challenges in a cell culture system (Agapito et al., 2010). Also, prenatal ethanol decreases *Per2* mRNA levels in the arcuate nucleus where many *POMC* neuronal cells are localized (Chen et al., 2006). Additionally, *Per2* gene is identified in laser captured microdissected β -endorphin neurons (Chen et al., 2006), indicating that POMC-producing neurons express the *Per2* gene. Also, a population of POMC neurons produces and releases glutamate (Hentges et al., 2009), which is also a target of *Per2* mutation (Spanagel et al., 2005).

How *Per2* gene mutation alters ethanol's action on POMC-producing neurons is not well understood at present. One possibility is that the *Per2* 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 *Bmal1* (Hiramaya 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, 2011). 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 et al., 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 et al., 2009). 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 (DiLorenzo 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., 2005). Furthermore, FASD patients are known to have altered sleep patterning (Burd et al., 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 ethanol-influenced expression of certain metabolic sensing genes (*Stat3*, *Sirt1*, *Pgc1 α* , and *Asb-4*) is regulated by *Per2*. 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 *Per2* gene mediates POMC neuronal functions and controlling ethanol action.

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Figure 1.

Effect of postnatal ethanol exposure on levels of *POMC* mRNA (A), β -endorphin (B) and α -MSH (C) and *Per2* (D) in the mediobasal hypothalamus (MBH) at PD7 and PD90 in C57BL/6 and *Per2*^{Brdml} Mice. Pups fed with milk formula containing alcohol (AF), pair-fed isocaloric milk formula (PF) or left in the litter (AD) between PD2–PD7. Data are mean $\pm$ SE. $N = 6$. Two-way ANOVA identified significant interaction between feeding effects and genotypes ($P < 0.05$). Bonferroni posttest identified differences between control groups (AD & PF) vs. AF treated group within the same mice strain (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$) or between C57BL/6 and *Per2* mutant mice in each feeding group (a, $P < 0.05$, aa, $P < 0.01$, aaa, $P < 0.001$).

Fig. 2.

Effect of postnatal ethanol exposure on mRNA levels of *Stat3* (A), *Sirt1* (B) and *Pgc1α* (C) and *Asb4* (D) in the mediobasal hypothalamus (MBH) at PD7 and PD90 in C57BL/6 and *Per2^{Brdml}* 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–PD7. Data are mean $\pm$ SE. $N = 6$. Two-way ANOVA identified significant interaction between feeding effects and genotypes ($P < 0.05$). Bonferroni posttest identified differences between control groups (AD & PF) vs. AF treated group within the same mice strain (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$) or between C57BL/6 and *Per2* mutant mice in each feeding group (a, $P < 0.05$, aa, $P < 0.01$, aaa, $P < 0.001$).