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Circadian rhythms, alcohol and gut interactions

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Abstract

The circadian clock establishes rhythms throughout the body with an approximately 24 hour period that affect expression of hundreds of genes. Epidemiological data reveal chronic circadian misalignment, common in our society, significantly increases the risk for a myriad of diseases, including cardiovascular disease, diabetes, cancer, infertility and gastrointestinal disease. Disruption of intestinal barrier function, also known as gut leakiness, is especially important in alcoholic liver disease (ALD). Several studies have shown that alcohol causes ALD in only a 20–30% subset of alcoholics. Thus, a better understanding is needed of why only a subset of alcoholics develops ALD. Compelling evidence shows that increased gut leakiness to microbial products and especially LPS play a critical role in the pathogenesis of ALD. *Clock* and other circadian clock genes have been shown to regulate lipid transport, motility and other gut functions. We hypothesized that one possible mechanism for alcohol-induced intestinal hyper-permeability is through disruption of central or peripheral (intestinal) circadian regulation. In support of this hypothesis, our recent data shows that disruption of circadian rhythms makes the gut more susceptible to injury. Our in vitro data show that alcohol stimulates increased Clock and Per2 circadian clock proteins and that siRNA knockdown of these proteins prevents alcohol-induced permeability. We also show that intestinal Cyp2e1-mediated oxidative stress is required for alcohol-induced upregulation of Clock and Per2 and intestinal hyperpermeability. Our mouse model of chronic alcohol feeding shows that circadian disruption through genetics (in ClockΔ^{19} mice) or environmental disruption by weekly 12h phase shifting results in gut leakiness alone and exacerbates alcohol-induced gut leakiness and liver pathology. Our data in human alcoholics show

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they exhibit abnormal melatonin profiles characteristic of circadian disruption. Taken together our data support circadian mechanisms for alcohol-induced gut leakiness that could provide new therapeutic targets for ALD.

Keywords

Intestinal permeability; Alcohol; Circadian rhythms; Per2; Cyp2e1; Dysbiosis

Circadian rhythms in our lives

The circadian clock establishes rhythms throughout the body with an approximately 24-h period; these rhythms are present at the cell, tissue, organ, and behavioral levels of organisms (Lowrey & Takahashi, 2011; Panda, Hogenesch, & Kay, 2002). The 24-h molecular clocks regulate the diurnal timing of the expression of hundreds of clock-controlled genes (Bozek et al., 2009; Lowrey & Takahashi, 2011; Panda, Antoch, et al., 2002). The circadian system enables the organism to synchronize to the external 24-h light/dark cycle, and also coordinates the timing of internal physiological systems necessary for optimal function. There is a central circadian clock located in the SCN in the hypothalamus that is entrained via light from the environment. In addition, there are also peripheral clocks in nearly all tissues and cells of the body including the liver, kidneys, heart, lungs, skeletal and smooth muscle, adipose tissue, and the intestines (Bell-Pedersen et al., 2005; Hastings, Reddy, & Maywood, 2003; Hoogerwerf et al., 2007; Sládek et al., 2007; Zvonic et al., 2006). Central and peripheral rhythms are generated by core molecular transcriptional-translational circadian regulatory feedback loops that cycle approximately once every 24 h (Mohawk, Green, & Takahashi, 2012). While the SCN is often considered the “conductor” of the “orchestra” of peripheral clocks, precisely how the central clock regulates the peripheral clocks is not yet entirely clear. Central regulatory circadian mechanisms that have been detailed include hormonal (e.g., melatonin), neural, and body temperature regulation. In addition to the central pacemaker, peripheral rhythms can also be entrained by environmental factors. For example, time of eating can alter the circadian clock in the intestine and liver, creating a situation where peripheral and central clocks are “uncoupled” or “misaligned” (Froy & Miskin, 2010; Malloy, Paulose, Li, & Cassone, 2012; Mohawk et al., 2012).

Chronic circadian rhythm disruption is a common feature of modern day society (Reddy & O'Neill, 2010). In humans, chronic circadian misalignment occurs most commonly in occupations that require working during the night or in the very early morning, such as in shift workers who make up 15–20% of the workforce (United States Department of Labor and Bureau of Labor Statistics, 2005), and also occurs in people who regularly cross multiple time zones, such as international flight crew (pilots, flight attendants) or even in the more common case of ‘social jetlag’ (Golombek et al., 2013; Roenneberg, Allebrandt, Merrow, & Vetter, 2012; Wittmann, Dinich, Merrow, & Roenneberg, 2006). Epidemiological data suggest this chronic circadian misalignment significantly increases the risk for a myriad of diseases, including cardiovascular disease, obesity, metabolic/diabetes, cancer, infertility, and gastrointestinal disease (Golombek et al., 2013; Knutsson, 2003;

Megdal, Kroenke, Laden, Pukkala, & Schernhammer, 2005; Monk & Buysse, 2013). Other factors that could also account for this increased risk for disease include, but are likely not limited to: poor diet, sleep disruption, melatonin suppression, and exposure to ionizing radiation at high altitude (Blask et al., 2005; Gangwisch et al., 2006; Knutson, Ryden, Mander, & Van Cauter, 2006; Sigurdardottir et al., 2013; Zeeb, Hammer, & Blettner, 2012). Nonetheless, laboratory studies in healthy humans have demonstrated that even short-term circadian misalignment increases metabolic, autonomic, and endocrine predictors of obesity, diabetes, and cardiovascular risk (Golombek et al., 2013; Scheer, Hilton, Mantzoros, & Shea, 2009).

Much less is known about the impact of circadian rhythm disruption on gastrointestinal health in humans, despite the fact that there is significant circadian regulation of digestive system activity (Hoogerwerf, 2009; Hoogerwerf et al., 2007, 2008; Polidarová, Sládek, Soták, Pácha, & Sumová, 2011; Scheving, 2000; Scheving & Russell, 2007). Disruption of gastrointestinal barrier function (by disease or environmental factors such as alcohol) can increase intestinal permeability (“gut leakiness”), thus enabling the translocation of bacterial products such as endotoxin, from the intestine into the circulation, triggering inflammatory cascades that can promote or exacerbate inflammatory-based diseases (Farhadi, Banan, Fields, & Keshavarzian, 2003; Turner, 2009; Wang, Gao, Zakhari, & Nagy, 2012). Epidemiological studies reveal higher rates of irritable bowel syndrome, gastric and duodenal ulcers, inflammatory bowel disease, and colorectal cancer in shift workers compared to daytime workers (Drake, Roehrs, Richardson, Walsh, & Roth, 2004; Knutsson, 2003; Nojkov, Rubenstein, Chey, & Hoogerwerf, 2010; Schernhammer et al., 2003; Segawa et al., 1987; Sonnenberg, 1990), and our recent publication demonstrates that circadian rhythm disruption in mice promotes intestinal hyper-permeability and exacerbates alcohol-induced intestinal hyper-permeability (Summa et al., 2013). Thus, circadian rhythm disruption may directly impact gastrointestinal health, but may also weaken or even play a role in the organism's response to injurious agents, such as promoting excessive alcohol consumption (Spanagel, 2009; Spanagel, Pendyala et al., 2005) or immune dys-regulation and inflammation (Curtis, Bellet, Sassone-Corsi, & O'Neill, 2014; Voigt, Forsyth, & Keshavarzian, 2013). Some potential mechanisms by which circadian disruption may adversely impact gastrointestinal health, especially through regulation of intestinal permeability, have been investigated in our *in vitro* studies using intestinal models as well as studies in animal models and alcoholics, as discussed below.

Alcohol, intestinal permeability, and disease

The intestinal tract has many important functions including the regulation of water balance and nutrient absorption, a significant role in immunity, and also forming a selective barrier to the proinflammatory microbial gut contents (Farhadi et al., 2003; Turner, 2009). Disruption of this barrier function, also known as gut leakiness, has been shown by many studies to be especially important in the pathogenesis of alcohol-induced pathologies and, in particular, alcoholic liver disease (ALD) (Bjarnason, Peters, & Wise, 1984; Keshavarzian et al., 1999; Purohit et al., 2008). Several epidemio-logical studies have shown that alcohol causes ALD in only a 20–30% subset of alcoholics (Grant, Dufour, & Harford, 1988; O'shea, Dasarathy, & McCullough, 2010). These data support the theory that alcohol is

required but is not sufficient for development of ALD. Thus, additional factor(s) that are involved in the pathogenesis of alcohol-associated pathologies such as ALD should be investigated because these factors could be ideal therapeutic targets for the prevention and/or treatment of pathologies in alcoholics. The underlying mechanisms for this differential susceptibility to alcohol-induced pathologies have not been well-established; however, multiple studies have demonstrated that inflammation and oxidative stress are required mechanisms for alcohol-induced pathologies, providing a strong rationale to examine the source of "sterile" inflammation in alcoholics (Wang, Zakhari, & Jung, 2010). This led to the discovery that LPS is required to induce alcohol-related tissue injury and pathologies like alcoholic liver disease (Adachi, Moore, Bradford, Gao, & Thurman, 1995; Keshavarzian et al., 2009; Wang et al., 2010). Since the intestinal microbiota is the primary source of LPS, our research group as well as others began to study the impact of alcohol consumption on intestinal barrier (permeability) function in rodent models of alcohol-induced pathologies and in human alcoholics. Studies found compelling evidence that increased gut leakiness to microbial products and especially LPS play a critical role in the pathogenesis of ALD (Bode & Bode, 2005; Enomoto et al., 2000; Parlesak, Schäfer, Schütz, Bode, & Bode, 2000). Our laboratory and others have shown that *in vitro*, animal, and human studies support the hypothesis that both alcohol and alcohol metabolites including acetaldehyde cause increased intestinal permeability ("leaky gut") and endotoxemia that drive ALD pathogenesis (Elamin, Masclee, Dekker, & Jonkers, 2013; Keshavarzian et al., 2009; Rao, 2009). Although alcohol uniformly causes leakiness in Caco-2 cell *in vitro* models of the intestine, only a subset of alcoholics that have intestinal hyperpermeability (20–30%) actually develop liver disease (Bode & Bode, 2005; Keshavarzian et al.,1999). Thus, gut leakiness to endotoxins (LPS) could be a susceptibility factor promoting alcohol-induced pathologies such as ALD in a subset of alcoholics. But what makes the intestine leaky after excessive consumption of alcohol in only a subset of alcoholics? The answer to this question and identifying the mechanisms through which alcohol promotes this gut leakiness could provide a tool for risk stratification/assessment as well as new avenues for prevention and/or treatment of ALD.

Circadian rhythms and intestinal injury

Several key points support a role for circadian regulation of intestinal permeability and possible pathology including: 1) The core circadian clock molecular machinery is within essentially all the tissues and organs of the body including the central circadian clock in the SCN, and intestinal epithelial cells (Hastings et al., 2003; Hoogerwerf et al., 2007; Mohawk et al., 2012; Yoo et al., 2004); 2) The SCN regulates and coordinates the expression and timing of peripheral circadian molecular rhythms, possibly including brain-gut interactions of the so-called "brain-gut axis" (BGA) (Bass & Takahashi, 2010; Mohawk et al., 2012); 3) The BGA can regulate intestinal permeability, and disruption of the BGA by physical and psychological stress can cause gut leakiness in both humans and rodents (Gareau, Jury, MacQueen, Sherman, & Perdue, 2007; Stasi & Orlandelli, 2008); 4) The circadian modulation of the brain-gut communication could mediate normal and pathological states of intestinal permeability because circadian genes regulate apical junctional complex (AJC) protein genes that are directly involved in regulation of intestinal permeability (Yamato et

al., 2010; Zelinski, Deibel, & McDonald, 2014); 5) Alcohol disrupts the central SCN circadian rhythm in rodents and affects expression of clock genes in the brain (Chen, Kuhn, Advis, & Sarkar, 2004; McElroy, Zakaria, Glass, & Prosser, 2009; Mistlberger & Nadeau, 1992; Rosenwasser, Fecteau, & Logan, 2005; Rosenwasser, Logan, & Fecteau, 2005; Seggio, Fixaris, Reed, Logan, & Rosenwasser, 2009; Spanagel, Rosenwasser, Schumann, & Sarkar, 2005); 6) *Clock* and other circadian clock genes have been shown to regulate lipid transport, motility, and other gut functions (Hoogerwerf, 2009; Hoogerwerf et al., 2010; Pan & Hussain, 2009); and 7) We have recently shown that disruption of circadian rhythms (light/dark phase shift) disrupts the intestinal microbiota community and causes dysbiosis in mice (Voigt et al., 2014) and it is well-established that the microbiota community affects and regulates intestinal barrier function (Forsyth et al., 2009; Frazier, DiBaise, & McClain, 2011; Hippe et al., 2014; Kerr et al., 2014; Mutlu et al., 2009, 2012). Thus, disruption of circadian rhythms would be expected to affect intestinal barrier function and furthermore, since alcohol disrupts the circadian clock alcohol-induced effects on circadian rhythms, may be further promoting intestinal barrier dysfunction.

We hypothesized that one possible mechanism for alcohol-induced intestinal hyperpermeability is through disruption of central or peripheral (intestinal) circadian regulation and that alcohol might cause significantly greater intestinal injury in the context of circadian disruption (either environmental or genetic) (Voigt et al., 2013). In support of this hypothesis, our recent data show that disruption of circadian rhythms by sleep deprivation or repeated L/D shifting makes the gut more susceptible to injury (Preuss et al., 2008; Tang, Preuss, Turek, Jakate, & Keshavarzian, 2009). In the sleep studies, mice subjected to sleep deprivation for 1 or 8 days exhibited significantly greater colon damage and colitis when given DSS in the drinking water (a well-validated model of colitis). However, the mice subjected to sleep deprivation alone did not demonstrate marked intestinal inflammation, histological evidence of colitis, or colon shortening (a measure of damage) (Tang, Preuss, et al., 2009). Similarly, in the L/D shifting studies, mice that were subjected to weekly 12-h light/dark (L/D) phase shifts for 12 weeks were found to exhibit more severe intestinal inflammation in the DSS model of colitis. In fact, the mortality of these mice was also increased. Interestingly, in those studies, the shifted mice that did not receive DSS in their drinking water also did not display abnormal histological intestinal measures (Preuss et al., 2008). The overall conclusion from these two initial intestinal circadian studies resulted in the hypothesis that circadian disruption alone has minimal effects on intestinal physiology but makes the intestines much more susceptible to injury and inflammation caused by a second stressor or challenge. Importantly, intestinal permeability was not measured in both of these studies and our later studies did find significantly increased intestinal permeability in control-fed animals after 12 weeks of L/D shifting. However, those later studies used a high sugar/high fat control diet (Lieber Decarli/Nanji) balanced for calories to match the high calorie liquid alcohol diet that is an appropriate control for the liquid alcohol diet but also appears to be a second stressor in itself. This will be discussed further below.

Alcohol, clock genes, and intestinal permeability

We sought to determine whether alcohol's effects on circadian clock gene expression and function might be involved in alcohol-induced gut leakiness. If true, disruption of circadian homeostasis could be one susceptibility factor for the development of gut leak-iness to LPS in a subset of alcoholics. For this investigation we used a well-established *in vitro* model of intestinal permeability (Caco-2 cell monolayers grown on permeable inserts), as well as tissue from mice fed a chronic alcohol diet in a rodent model of alcoholic steatohepatitis (a form of ALD). Using Caco-2 cells (intestinal epithelial cells), we first sought to establish that physiological levels of alcohol found in the blood in the distal small intestine and colon (0–0.5%) cause intestinal permeability (Swanson et al., 2011). Our data show that alcohol (0.1%–0.5%) causes a dose- and time-dependent increase in intestinal permeability to the fluorescent dye FSA (Swanson et al., 2011) and an alcohol concentration of 0.2% (43 mM, 2–4 drinks) causes a decrease in trans-epithelial resistance (TER), another measure of intestinal permeability in this model. To evaluate the mechanisms behind this phenomenon we selected 0.2% alcohol concentration because it represents blood alcohol values found in the colon of humans and blood from our rodent model of ALD (Halsted, Robles, & Mezey, 1973; Keshavarzian et al., 2001). We used Western blots of Caco-2 cells exposed to 0.2% alcohol to measure levels of the canonical clock gene proteins CLOCK and PER2 (Swanson et al., 2011). Cell lysates were made from the same membrane-grown Caco-2 cells that were tested for alcohol-induced permeability. CLOCK and PER2 protein levels remained virtually unchanged over 4 h in control cells while, in contrast, CLOCK and PER2 proteins were increased in response to alcohol, an effect that was observed as early as 30 min after alcohol exposure and significantly increased after 60 and 240 min. Compared to time point-matched controls, over the 4-h period alcohol treatment resulted in a PER2 protein increase of 315% and CLOCK protein increase of 237%. The increases in these proteins correlate with our measures of alcohol-induced intestinal hyper-permeability assessed via FSA flux and TER. We next used siRNA, specific for Clock and Per2 in the same Caco-2 model, and found that siRNA knockdown of either CLOCK or PER2 protein expression significantly prevented alcohol-induced permeability of the monolayers using both FSA and TER measures of permeability (Swanson et al., 2011). Thus, alcohol-induced upregulation of CLOCK and PER2 proteins is required for alcohol-induced permeability of Caco-2 monolayers. It should be noted that Clock is a transcription factor that activates Per2 expression (Mohawk et al., 2012). We then sought to determine if the same effect could be observed *in vivo* in our rat model of chronic alcohol consumption, in which rats were gavaged once daily with 6 g/kg/day alcohol for 10 weeks. This alcohol treatment protocol significantly increases intestinal permeability assessed using urinary sucralose measured via gas chromatography after sugar gavage (Farhadi, Keshavarzian, Fields, Sheikh, & Banan, 2006; Keshavarzian et al., 2001). These rats also exhibited endotoxemia and steatohepatitis (Keshavarzian et al., 2001) and dysbiosis of the intestinal microbiota (Mutlu et al., 2009). Western blot analysis of duodenum and colon intestinal tissue from alcohol-fed and control rats revealed that alcohol significantly increases PER2 protein in the intestinal tissues from rats that demonstrated increased gut permeability (Swanson et al., 2011). One intriguing idea is that probiotic gavage with daily *Lactobacillus GG* resulted in amelioration of dysbiosis and gut leakiness and markers of intestinal oxidative stress, but we have not yet determined the

effects of probiotic treatment on Per2 expression in these animals (Forsyth et al., 2009). Thus, our *in vivo* data support the *in vitro* Caco-2 cell data and support a role for alcohol stimulation of PER2 intestinal clock gene protein expression in alcohol-induced intestinal permeability.

Role for intestinal Cyp2e1 in alcohol/circadian effects

We sought to identify a mechanism through which alcohol might stimulate the CLOCK and PER2 circadian clock gene protein expression observed in Caco-2 cells and in the intestines of alcohol-fed rats. Several studies of clock gene regulation have shown that oxidative stress results in stimulation of transcription of clock genes that contain a redox-sensitive PAS domain, including Clock and Per2 (Hirayama, Cho, & Sassone-Corsi, 2007; Loudon, 2012; Rutter, Reick, & McKnight, 2002; Rutter, Reick, Wu, & McKnight, 2001; Tamaru et al., 2013; Taylor & Zhulin, 1999). Many of the pathological effects of alcohol have been tied to oxidative stress; thus, we hypothesized this might be a key mechanism influencing clock gene expression (Wilking, Ndiaye, Mukhtar, & Ahmad, 2012). One major element in alcohol-mediated oxidative damage to the liver is the metabolism of alcohol by cytochrome P450 2E1 (CYP2E1) (Lieber, 2004; Lu & Cederbaum, 2008, 2010; Lu, Wu, Wang, Ward, & Cederbaum, 2010). Studies had shown that CYP2E1 is also expressed in the intestinal epithelium (Bergheim, Bode, & Parlesak, 2005; Roberts, Shoaf, Jeong, & Song, 1994); however, the role of CYP2E1 or CYP2E1-generated oxidative stress in alcohol-induced intestinal permeability had not been examined. Using our *in vitro* Caco-2 cell model of intestinal permeability, we sought to determine if intestinal CYP2E1-mediated oxidative stress might be contributing to alcohol-induced clock gene protein expression and intestinal permeability (Forsyth et al., 2013). For these studies, Caco-2 cells were treated with 0.2% alcohol, and CYP2E1 mRNA, protein, and enzymatic activity were examined. We found exposure to alcohol for 4 h does not increase CYP2E1 mRNA, but does increase CYP2E1 protein by 93% and activity by 69%. This agrees with data from other investigators for alcohol treatment of liver cells (Roberts, Song, Soh, Park, & Shoaf, 1995) and intestinal Cyp2e1 in alcohol-fed mice (Roberts et al., 1994). In addition, we examined Cyp2e1 mRNA and protein levels in colon tissue collected from BL/6 mice fed a chronic alcohol-containing diet (Tipoe et al., 2008) which contained 4.5% alcohol for 8 weeks. As was the case *in vitro*, there was no increase in Cyp2e1 mRNA but CYP2E1 protein increased by 73% in colonic tissue. Importantly, knockdown of CYP2E1 via siRNA protects Caco-2 cells from alcohol-induced increases in CLOCK and PER2 proteins and prevents alcohol-induced permeability. In addition, pretreatment of Caco-2 cells with the antioxidant N-acetylcysteine (NAC) significantly blocks the alcohol-induced increase in CLOCK and PER2 proteins as well as alcohol-induced hyper-permeability. In further support of an oxidative stress mechanism, the effects of alcohol on CLOCK and PER2 were mimicked with H_2O_2, an effect that we also blocked with NAC. These data support the hypothesis that alcohol-induced oxidative stress due to alcohol metabolism by intestinal CYP2E1 and the resulting upregulation of CLOCK and PER2 circadian gene proteins is a novel circadian-based mechanism for alcohol-induced intestinal permeability (Forsyth et al., 2013). Data recently published in collaboration with researchers at the NIH support this model in Cyp2e1 KO mice and a binge model of alcohol feeding. In those studies, Cyp2e1 KO mice or WT mice treated with the Cyp2e1 inhibitor

chlormethiazole or the antioxidant N-acetylcysteine have blunted alcohol-induced intestinal permeability, endotoxemia, and hepatic steatosis and inflammation (Abdelmegeed et al., 2013). The WT alcohol-fed mice exhibited increased serum TNF-α, hepatic cytokines, CYP2E1 protein, and lipid peroxidation, with decreased levels of mitochondrial superoxide dismutase and suppressed aldehyde dehydrogenase 2 activity. In the livers of these WT alcohol-fed mice, increased hepatocyte apoptosis with elevated levels of proapoptotic proteins and decreased levels of active (phosphorylated) p-AKT, p-AMPK, and peroxisome proliferator-activated receptor-α were observed, all of which are involved in fat metabolism and inflammation. These markers were all diminished in the alcohol-fed Cyp2e1 KO mice. Thus, Cyp2e1 appears to play a key role not only in alcohol-induced liver injury, but also in intestinal leakiness to microbial contents needed for ALD. Thus, these *in vitro* (Forsyth et al., 2013) and *in vivo* (Abdelmegeed et al., 2013) data support our hypothesis that intestinal CYP2E1 is contributing to alcohol-induced effects on the intestine, possibly via a circadian mechanism.

Circadian disruption, alcohol, and ALD

Because of our *in vitro* data, we hypothesized that circadian mechanisms regulate intestinal permeability, and that alcohol-induced disruption of circadian rhythms or environmental/genetic circadian rhythm disruption or both together may be a significant mechanism contributing to gut leakiness and ALD (Fig. 1). We set out to test this hypothesis *in vivo* using two models of circadian disruption in mice fed a chronic alcohol-containing diet (4.5% v/v alcohol) for 8 weeks with an additional 2-week buildup of alcohol dose (control mice were fed a calorically matched diet with alcohol calories replaced with dextrose) (Summa et al., 2013). Our first model was a genetic circadian disruption model using ClockΔ^{19} mutant mice that have been widely used to investigate circadian mechanisms in physiology (Vitaterna et al., 1994). ClockΔ^{19} mice are not knockout mice, but instead express a dominant negative mutant form of the core circadian transcription factor called Clock. Homozygous Clock$\Delta^{19/}\Delta^{19}$ mice exhibit a variety of symptoms characteristic of circadian disruption, including obesity and metabolic syndrome (Turek et al., 2005), reproductive dysfunction (Miller et al., 2004), and behavioral disorders (McClung, 2007). Wild-type littermates were used as controls. Our second model was an environmental circadian rhythm disruption model, subjecting WT-BL/6 mice to once-weekly 12-h light:dark phase shifts for 12 weeks before the 10 weeks of alcohol diet and then continuing throughout the alcohol diet feeding until sacrifice. We used orally administered sugars and gas chromatographic analysis of these sugars in 5-h urine samples to measure intestinal permeability in these mice before and after chronic alcohol consumption (Summa et al., 2013). We found that both genetic and environmental circa-dian rhythm disruption promote intestinal barrier dysfunction, alcohol-induced intestinal hyperpermeability, and alcoholic steatohepatitis (Summa et al., 2013). Intestinal permeability is significantly higher in ClockΔ^{19} mice without alcohol consumption. Intestinal permeability was also significantly greater in the environmentally disrupted mice that consumed no alcohol. However, as noted above, the calorie-matched control diet for these experiments is well-validated in many Lieber-Decarli alcohol diet studies but contains high sugar and fat, thus modeling the classic Western diet that has now been shown to be pro-inflammatory (Huang, Devkota, Moscoso,

Chang, & Leone, 2013; Manzel et al., 2014). Studies are now underway in our laboratory to compare these permeability and inflammation data to similarly shifted mice given a standard chow diet and water. Thus, circadian disruption alone promotes gut leakiness but with the caveat of the high calorie diet. In the alcohol-fed mice, both the ClockΔ^{19} mice and the environmentally disrupted mice demonstrated exacerbated alcohol-induced intestinal hyperpermeability compared to the mice that received alcohol diet alone, as well as, of course, the control-fed mice. Analysis of 24-h circadian patterns of the tight junction protein occludin (one regulator of gut permeability) in intestinal tissue suggests that circadian rhythm disruption with or without alcohol alters circadian occludin expression, which may be one mechanism accounting for the observed effects on intestinal permeability. In the control-fed ClockΔ^{19} mice, liver steatosis was increased compared to WT controls, and in the environmentally disrupted mice, both liver inflammation and steatosis were significantly increased compared to non-shifted control-fed mice. Importantly, the alcohol-fed environmentally disrupted mice developed more severe steatohepatitis compared to non-shifted alcohol-fed mice and hepatocyte necrosis was observed only in environmentally disrupted alcohol-fed mice (Summa et al., 2013). In contrast to the alcohol-fed phase-shifted mice, alcohol-fed ClockΔ^{19} mice did not develop hepatic inflammation in spite of gut leakiness and endotoxemia. However, these mice had more severe steatosis than wild type alcohol-fed mice, an effect that may be the consequence of ClockΔ^{19} mice having resistance to LPS effects and resistance to LPS-induced activation of the NF-kB pathway (Bellet et al., 2013; Curtis et al., 2014), as well as problems with fat metabolism in ClockΔ^{19} mice (Turek et al., 2005). Thus, it appears that disruption of the sleep/wake cycle (circadian) pattern that is commonly seen in a subset of alcoholics may prime the intestine to the injurious effects of alcohol, leading to gut leakiness, endotoxemia, and steatohepatitis. Indeed, our recently generated data in alcoholics showed that there is a significant inverse correlation between the severity of gut leakiness (defined by 24-h urinary sucralose) and 24-h serum melatonin in alcoholics, suggesting that disruption of normal circadian output may promote gut leakiness in alcoholics (Swanson et al., 2014).

Implications and future directions

Our early experiments led to the hypothesis that circadian disruption alone did not produce overt gastrointestinal pathology, but instead made the mammalian system more susceptible to a second hit or stressor such as alcohol (Preuss et al., 2008; Tang, Preuss, et al., 2009), but can we say this is the case? The answer is we still cannot be certain, because our control diet in the shifting experiments was a high-fat and high-sugar calorie-matched diet to match the liquid alcohol-diet calories as is standard for the Lieber-Decarli diet. Circadian disruption by phase shifting alone with this high-fat, high-sugar diet did result in increased intestinal permeability and liver pathology mimicking non-alcoholic steatohepatitis (NASH), as a consequence of phase shift or fatty liver (steatosis) caused by a genetic mutation (as in the ClockΔ^{19} mutant mice). Experiments are now underway to test the effects of a standard chow diet in these circadian disruption models. Our data support the hypothesis that there is a complex interplay between circadian rhythm disruption and alcohol-induced effects. This is not surprising because disruption of circadian homeostasis could have a profound impact on organ function, such as the intestines and liver, based on whether the rhythm is disrupted

and/or whether rhythm and core circadian genes and circadian-controlled genes are mis-aligned and dysfunctional. Indeed, approximately 10% of the genes in any one tissue are under circadian control and these differ from tissue to tissue; therefore, the variability observed with our models of circadian rhythm disruption may be the consequence of disrupting the circadian clock genes (and related circadian-controlled genes) in a tissue-specific manner (Bozek et al., 2009; Panda, Antoch, et al., 2002). Specifically, the ClockΔ^{19} mutant mouse has mutated clock protein in every cell in the body, whereas the environmental circadian rhythm disruption would be expected to have differential effects in the brain vs. the intestine vs. the liver, thus creating misalignment between central and peripheral circadian machinery that would have different biological effects compared to a global circadian gene dysfunction. Our data indicate that the type of circadian disruption may result in specific dysfunction, emphasizing the importance of studying the effects of different types of circadian disruption [e.g., light/dark phase shift, genetic manipulation of different circadian genes] on alcoholic-associated diseases such as alcohol-induced intestinal function, liver disease, myopathy, cardiomyopathy, and CNS injury – diseases that are all inflammation-mediated disorders. Experiments are now underway in our laboratory with environmental and genetic models of circadian rhythm disruption in combination with alcohol consumption analysis of gene expression in individual tissues, and we predict each type of circadian rhythm disruption approach with/ without alcohol will have its own unique profile of dysfunction. This view is supported by genetic data in mice and humans demonstrating dramatically different phenotypes depending on the specific type of genetic circadian clock abnormality (Arble, Ramsey, Bass, & Turek, 2010; Maury, Ramsey, & Bass, 2010).

To what extent do circadian mechanisms and alcohol operate through similar or different mechanisms? Our data support that both circadian rhythm disruption and alcohol consumption each have their own mechanisms that may synergize when combined. Both occludin and claudin-1 (tight junction proteins critical for regulating intestinal permeability) are down-regulated as a consequence of chronic alcohol consumption (Summa et al., 2013), and yet both of these proteins are also under direct circadian control (Kyoko et al., 2014), indicating a point of convergence between alcohol and circadian-mediated effects. However, only some alcohol effects are circadian-mediated. Our laboratory has published extensively regarding the roles of non-circadian mechanisms of alcohol regulation of gut permeability and injury including transcription factors (e.g., NF-kB, Snail) as well as iNOS (Banan et al., 2007; Forsyth, Tang, Shaikh, Zhang, & Keshavarzian, 2011; Tang, Forsyth et al., 2009). Further studies are necessary to fully elucidate the overlapping and unique aspects of circadian rhythm disruption and alcohol consumption.

We have recently demonstrated that circadian rhythm disruption changes the intestinal microbiota (Voigt et al., 2014). Changes in the microbiota have been associated with both intestinal hyper-permeability (Frazier et al., 2011) as well as steatohepatitis in alcohol-fed rats and ALD in humans reported by us and others (Chen & Schnabl, 2014; Mutlu et al., 2009, 2012). Studies have shown an interplay between the intestinal microbiota and circadian rhythms (Mukherji, Kobiita, Ye, & Chambon, 2013); thus, intestinal dysbiosis may be an additional mechanism through which circadian rhythms and alcohol may interact to affect intestinal pathology. It is unclear if changes in the microbiota precede or are a

consequence of other circadian-induced changes in the host, but this intriguing possibility needs to be further explored (summarized in Fig. 2).

Experiments are underway in our laboratory analyzing gene expression in intestinal and liver tissue from alcohol-fed, circadian-disrupted mice to determine how alcohol consumption and circa-dian rhythm disruption may be similar or dissimilar on the molecular level, indicating potential molecular points of synergism between these two conditions. In addition, alcohol exerts robust epigenetic effects on gene regulation (Mead & Sarkar, 2014) as do circadian mechanisms (Orozco-Solis & Sassone-Corsi, 2014); thus, we are evaluating differences and points of overlap with regard to gene acetylation and methylation. We predict that circadian rhythm disruption and chronic alcohol consumption will exhibit both a common as well as a unique profile of affected genes. However, mRNA expression may not be the best approach to fully characterize effects. mRNA expression does not tell the whole story of circadian regulation, as has been noted recently (Koike et al., 2012), and our laboratory has demonstrated that while the mRNA of the tight junction protein occludin was not indicative of intestinal permeability, occludin protein localization data did mirror the combined alcohol and circadian effects on intestinal permeability, with the greatest effect being with circadian disruption and alcohol combined (Summa et al., 2013). Thus, in order to gain a comprehensive understanding of points of intersection between alcohol and circadian rhythm disruption, detailed analysis of proteins and localization of those proteins will likely be required.

Potential translational impact

Another important implication of our studies is the potential translational impact. In an overall view, our studies and those of others highlight strong circadian-alcohol interactions at molecular and behavioral levels and thus support a need for consideration of circadian factors in the treatment of chronic alcohol abuse. A straightforward approach would be to try to minimize circadian disruption in the lifestyle and eating patterns of persons with alcohol-use disorders. The degree of circadian disruption (sleep, eating patterns, and even drinking patterns) could be assessed and used for risk stratification and possible chrono-therapeutic measures. Chronotherapy and chronopharmacology have been shown to be effective in cancer treatment, mental illness, sleep disorders, and several other diseases (Coogan & Thome, 2011; Innominato, Lévi, & Bjarnason, 2010; Kaur, Phillips, Wong, & Saini, 2013). However, to our knowledge chronotherapy has not been applied to treatment of alcohol abuse, although Cyp2e1 is also under circadian control (Košsir, Španinger, & Rozman, 2013).

In addition, our studies with alcohol-mediated effects on circadian clock proteins, Cyp2e1, and intestinal permeability support that one mechanism for alcohol-mediated interaction with circadian rhythms is through oxidative stress. Others have shown that the circadian clock is redox sensitive (Rutter et al., 2001, 2002) and N-acetylcysteine prevented alcohol-induced effects on Clock and Per2 proteins in our studies (Forsyth et al., 2013). Therefore, agents that ameliorate oxidative stress could potentially be used to prevent alcohol-induced circadian-mediated effects on the intestines and other organs.

Finally, we and others have highlighted the importance of gut-derived LPS in alcohol-induced organ damage. Thus, intervention that could ameliorate dysbiosis and fortify intestinal barrier integrity has the potential to mitigate the negative impact of disrupted circadian homeostasis and alcohol-induced organ damage. Indeed, we and others have shown that probiotics and oat prebiotics can reduce intestinal oxidative stress and improve intestinal permeability and can also correct dysbiosis due to chronic alcohol use in alcohol-fed rodents and alcoholics (Forsyth et al., 2009; Keshavarzian et al., 2001; Kirpich et al., 2008; Wang et al., 2011). Thus not only probiotics but also prebiotics and symbiotics, that are capable of limiting endotoxemia by modifying intestinal microbiota and by improving intestinal barrier function, could have applications in treatment of alcohol-induced intestinal circadian disruption and prevention/treatment of alcohol-induced organ damage. We are currently examining this possibility in our animal models.

In summary, additional models to investigate the relationship between circadian rhythms and alcohol in intestinal, liver, and brain function (as well as other organs) are currently underway in our laboratory. We are examining the direct effects of alcohol on circadian gene expression as well as expression of other genes, including those regulating inflammation/immunity, metabolism, and permeability functions in circadian-alcohol models. We believe that a better understanding of these mechanisms and effects will shed light on understanding the complex relationship that has emerged between circadian rhythms and alcohol. This understanding is expected to lead to new therapies for ALD and other alcohol-related pathologies.

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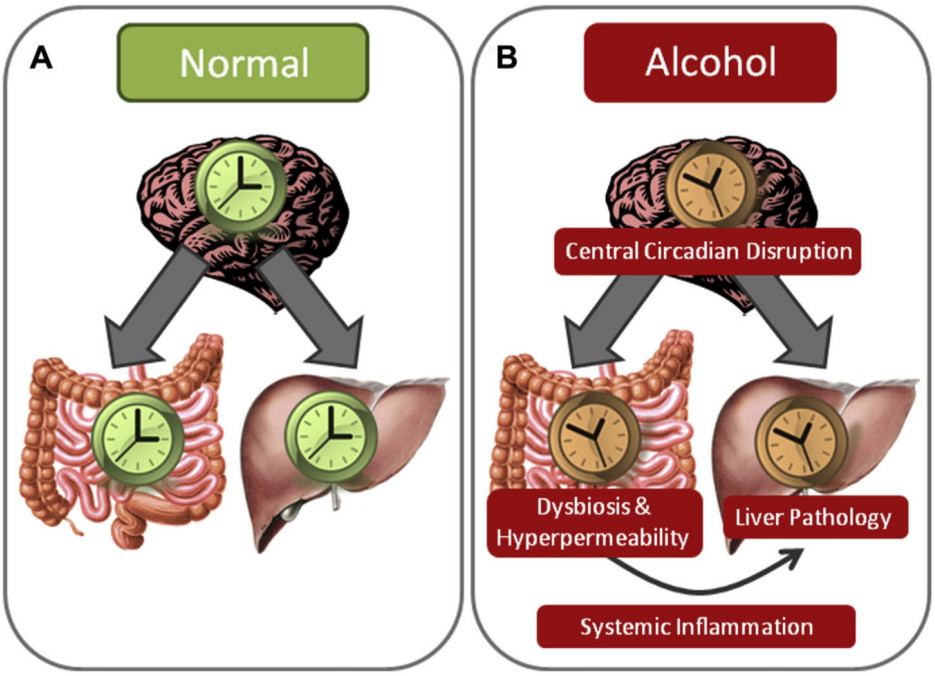

Fig. 1.

Alcohol and circadian disruption combine to promote alcoholic liver pathology and systemic inflammation through effects on the gut, including hyperpermeability and dysbiosis.

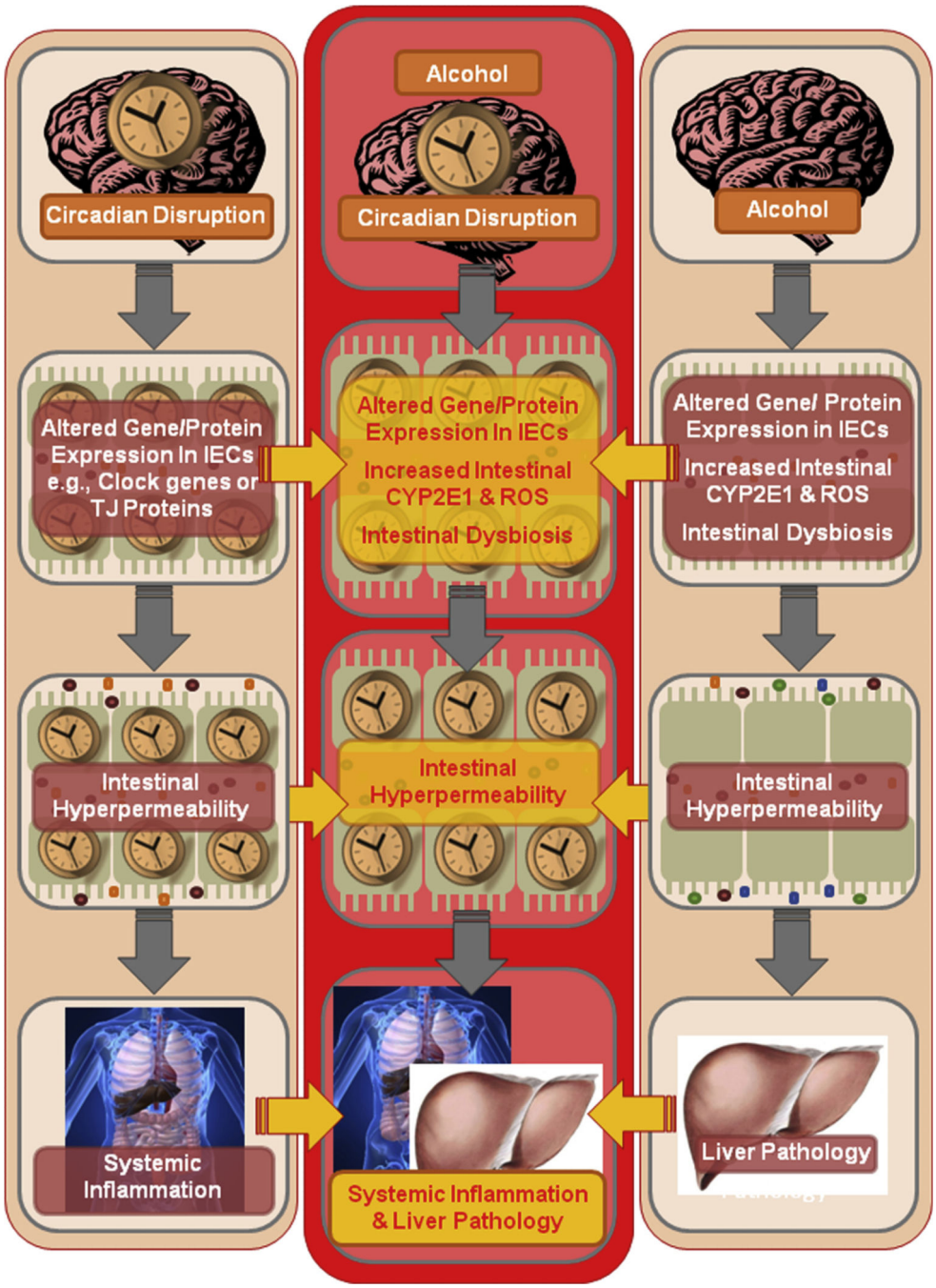

Fig. 2.

Possible mechanisms of alcohol- and circadian disruption-induced gut leakiness include dysbiosis of gut bacteria and increased expression of Cyp2e1, resulting in oxidative stress (ROS) and changes to intestinal epithelial cell (IEC) circadian and junctional proteins, resulting in intestinal hyperpermeability to gut microbial contents such as LPS. Dysbiosis may also contribute to inflammation. These combine to promote systemic inflammation and liver pathology.