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Chronic Alcohol Exposure and the Circadian *Clock* Mutation Exert Tissue-Specific Effects on Gene Expression in Mouse Hippocampus, Liver and Proximal Colon

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Abstract

Background—Chronic alcohol exposure exerts numerous adverse effects, although the specific mechanisms underlying these negative effects on different tissues are not completely understood. Alcohol also affects core properties of the circadian clock system and it has been shown that disruption of circadian rhythms confers vulnerability to alcohol-induced pathology of the gastrointestinal barrier and liver. Despite these findings, little is known of the molecular interactions between alcohol and the circadian clock system, especially regarding implications for tissue-specific susceptibility to alcohol pathologies. The aim of the present study was to identify changes in expression of genes relevant to alcohol pathologies and circadian clock function in different tissues in response to chronic alcohol intake.

Methods—Wild-type and circadian *Clock*$^{\Delta 19}$ mutant mice were subjected to a 10-week chronic alcohol protocol, after which hippocampal, liver and proximal colon tissues were harvested for gene expression analysis using a custom-designed multiplex magnetic bead hybridization assay that provided quantitative assessment of 80 mRNA targets of interest, including five housekeeping genes and a predetermined set of 75 genes relevant for alcohol pathology and circadian clock function.

Results—Significant alterations in expression levels attributable to genotype, alcohol and/or a genotype by alcohol interaction were observed in all three tissues, with distinct patterns of expression changes observed in each. Of particular interest was the finding that a high proportion of genes involved in inflammation and metabolism on the array was significantly affected by

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alcohol and the *Clock*$^{\Delta 19}$ mutation in the hippocampus, suggesting a suite of molecular changes that may contribute to pathological change.

Conclusions—These results reveal the tissue-specific nature of gene expression responses to chronic alcohol exposure and the *Clock*$^{\Delta 19}$ mutation, and identify specific expression profiles that may contribute to tissue-specific vulnerability to alcohol-induced injury in the brain, colon and liver.

Keywords

Circadian rhythms; Alcohol; Gene expression; Mouse models; Clock mutation

Introduction

Chronic alcohol consumption is associated with significant mortality and substantial medical, neurological and psychiatric co-morbidity, including steatohepatitis and cirrhosis, pancreatitis, cardiomyopathy, increased risk of hypertension, several cancers (e.g., head and neck, esophagus, colon, pancreas and liver), cognitive impairment, mood disorders and depression (Gao and Bataller, 2011, Kelley and Dantzer, 2011). Interestingly, not all alcoholics develop organ damage (Grant et al., 1988, Keshavarzian et al., 1999). Furthermore, of those alcoholics that do exhibit some degree of alcohol-related toxicity, not all organs are susceptible (Wang et al., 2010, Szabo and Bala, 2010). As such, we sought to understand tissue-specific responses to alcohol by examining three different tissues that are affected by alcohol: hippocampus, liver and colon. These tissues were studied because chronic alcohol exposure contributes to pathological damage and/or functional impairment in each.

The genes examined in each organ encompass mechanisms believed to contribute to the pathogenesis of alcohol-related tissue injury, including inflammation, metabolism, oxidative stress, cell-cell junction and epithelial permeability, brain-gut axis, cell division/cancer and circadian rhythms and circadian clock-controlled genes, to allow us to determine whether these organs affected by alcohol exhibit similar transcriptional profiles. Although broad associations between chronic alcohol consumption and subsequent biological responses have been made, the precise molecular mechanisms leading to alcohol-induced physiological dysfunction, tissue injury and organ dysfunction remain incompletely understood, and we sought to fill this gap in knowledge.

The circadian clock system represents a biological mechanism that may contribute to alcohol-induced tissue injury and organ damage because: (1) disruption of the circadian clock system promotes inflammation (Castanon-Cervantes et al., 2010), and (2) alcohol can impact the circadian clock in different organs (Chen et al., 2004, Spanagel et al., 2005b, Summa et al., 2013, Swanson et al., 2011). Studies using Caco-2 cells, a human epithelial colorectal adenocarcinoma cell line that serves as an *in vitro* model of intestinal barrier function, have demonstrated that circadian clock genes influence alcohol-induced increases in monolayer permeability: knock-down of the circadian genes *Clock* and *Per2* prevents alcohol-induced increases in permeability, indicating that these genes respond to alcohol and impact the regulation of barrier integrity (Swanson et al., 2011). Additionally, our recent

studies have shown that disruption of circadian organization in mice, using independent genetic and environmental strategies, exacerbates alcohol-induced intestinal hyperpermeability and hepatic pathology (Summa et al., 2013). Together, these results suggest complex relationships between the circadian clock system and biological responses to alcohol, however the mechanisms through which alcohol and the circadian system interact to promote tissue injury are not fully understood.

A number of studies have examined how alcohol impacts core properties of the circadian clock system, such as the free-running period, the phase of entrainment and the phase-shifting effects of exposure to light pulses (Brager et al., 2011a, Brager et al., 2010, Brager et al., 2011b, Rosenwasser et al., 2005). There has also been significant interest in identifying and cataloging diurnal and circadian gene expression rhythms, as well as identifying downstream targets and gene networks affected by the circadian clock in various mouse tissues and genetic models, albeit in the absence of alcohol (Miller et al., 2007, Panda et al., 2002, Storch et al., 2002). However, relatively little effort has been made in linking alcohol consumption and circadian rhythm disruption, especially in different genetic models of circadian disruption. Besides liver, other tissues and brain regions most vulnerable to alcohol-related damage have not been well-studied. Therefore not much is known regarding the specific molecular mechanisms underlying the impact of alcohol on circadian clock gene expression in different tissues or on the regulation of molecular/cellular pathways relevant to alcohol-related pathology in mouse models of circadian disruption.

We attempted to understand the relationships between alcohol, alcohol-related pathologies, the circadian clock, and the regulation of gene expression by examining expression patterns in the hippocampus, liver and colon of alcohol-fed or alcohol-free control diet-fed wild-type mice and circadian *Clock*$^{\Delta 19}$ mutant mice (*Clock* mutant hereafter). Hippocampus, liver and colon were examined in the current study because chronic alcohol exposure contributes to pathological damage and/or functional impairment in each tissue. The results presented in this study were obtained from analyses of tissues collected from mice originally described in a previous publication reporting *in vivo* physiological measurements as well as post-mortem tissue analyses (Summa et al., 2013). Mutant mice harbor a dominant-negative mutation (Δ19) of the core circadian gene *Clock* (*Clock* mutation hereafter) (King et al., 1997a, King et al., 1997b, Vitaterna et al., 1994). *Clock* mutant mice were utilized as a model of circadian clock disruption to determine whether this particular genetic aberration of the clock affects the regulation of gene expression in response to chronic alcohol consumption in tissues susceptible to alcohol toxicity. These animals have been used widely to study the role of the circadian clock system in various physiological and pathophysiological processes (Naylor et al., 2000, Roybal et al., 2007, Summa et al., 2013, Turek et al., 2005).

We examined the expression of 75 genes (inflammation, metabolism, oxidative stress, cell-cell junction and epithelial permeability, brain-gut axis, cell division/cancer and circadian rhythms and circadian clock-controlled genes) and identified tissue-specific alterations in expression from each functional gene category. Distinct sets of genes were identified that were significantly affected by genotype, alcohol or genotype by alcohol interaction. Such alterations may contribute to pathological transformation in tissues affected by chronic

alcohol exposure and may underlie, at least in part, tissue-specific vulnerability to alcohol toxicity and injury.

Materials and Methods

Laboratory Animals

Young adult (7-9 week old) male mice homozygous *Clock* mutant mice and wild-type littermate mice, obtained from the breeding colony maintained at Northwestern University, were individually housed in light-tight, ventilated cabinets and maintained on a constant 12 hours light:12 hours dark (12:12 LD) cycle at constant temperature and humidity, as described previously (Summa et al., 2013). Locomotor activity rhythms were measured as previously described using cages equipped with infrared beams that cross through the cage and record breaks in the beam as activity (Summa et al., 2013). Upon separation into individual cages, mice were assigned to either the alcohol experimental group or the alcohol-free, dextrose control group for the duration of the experimental protocol described below. Group selection was determined by randomly assigning an individual mouse of each genotype to each dietary group (i.e., alcohol or dextrose control), then matching subsequent animals by body weight such that there were no significant differences in body weight between groups at the onset of the experiment. The experimental design included four groups: 1) Wild-type dextrose diet (WTD), wild-type alcohol diet (WTA), *Clock* mutant dextrose diet (CD) and *Clock* mutant alcohol diet (CA). All studies were reviewed and approved in advance by the Institutional Animal Care and Use Committee at Northwestern University.

Experimental Protocol

The experiments utilized the Nanji liquid alcohol diet protocol as described previously (Nanji et al., 1994, Summa et al., 2013). This diet, used to study alcoholic steatohepatitis in rodents (Forsyth et al., 2011, Summa et al., 2013), is a modification of the Lieber-DiCarli rodent alcohol diet. The Nanji diet utilized in the present study is nutritionally balanced, prepared fresh daily and provided to the mice at Zeitgeber Time 4 (i.e., ZT4, four hours after light onset) in specialized individual feeding tubes that allow for daily monitoring of food intake, which was monitored by checking diet levels at light onset and light offset for one or two consecutive 24-hour periods each week of the experimental protocol. There was a two-week gradual introduction to alcohol in the diet (0-29% of total calories from alcohol), followed by eight weeks on the full alcohol-containing diet (29% of total calories from alcohol, 4.5% v/v).

All mice were euthanized by conscious decapitation and tissues were harvested at ZT6 for gene expression analyses. The hippocampus was dissected over dry ice. Isolated hippocampus, liver and proximal colon samples were immediately frozen in liquid nitrogen and stored at -80°C until RNA isolation and subsequent gene expression analysis.

The tissues were harvested for expression analysis at ZT6 because we observed that mice were reliably consuming relatively large portions of their daily diet intake during the light phase after placement of the freshly prepared diet into each animal's home cage daily at ZT4

(Supporting Online Information). Thus, collection at ZT6 was presumed to enable assessment of expression changes occurring near the onset of a predictable daily bout of drinking. In addition, we have data for serum alcohol levels at ZT6 (Summa et al., 2013) and preliminary data suggesting that intestinal permeability peaks near ZT0 (data not shown), therefore we felt that analyzing expression at ZT6 may incorporate biological responses occurring near the apparent peak in daily endotoxin levels.

Assessment and Analysis of Gene Expression

Gene expression analysis was performed at the University of Illinois-Chicago core facility using an Affymetrix (Santa Clara, CA) custom QuantiGene 80 Plex assay. Briefly, this assay provides quantitative measurement of 80 mRNA targets of interest on a single plate. The 80 genes measured in this study included five housekeeping genes and a set of 75 genes selected from nine functional categories, including inflammation, metabolism, oxidative stress, cell-junction and epithelial permeability, brain-gut axis, cell division/cancer and circadian rhythms and circadian clock-controlled genes (CCGs) (Table 1).

Hippocampus, liver and proximal colon samples were prepared as tissue homogenates in our laboratory using Affymetrix lysis buffer and processed according to the manufacturer's instructions. Raw intensity readings of RNA amount were obtained for subsequent statistical analyses. These values were processed and analyzed as described in the Supporting Online Information. In order to validate results obtained from the analysis of the array expression data, we performed RT-PCR on *Tlr9* in the hippocampus, *Tlr5* in the liver and *Cdh1* in the proximal colon, as described in the Supporting Online Information.

Results

Hippocampus

In total, 56 genes exhibited significantly altered expression patterns in the hippocampus for at least one term (genotype, alcohol or genotype by alcohol interaction) among experimental groups. These genes group into four clusters (i.e., orange, blue, pink and green) based on the similarity of expression changes (Table 2, Figure 1).

Genotype—Genes in the orange cluster were predominantly influenced by genotype. This cluster includes 18 genes, the expression levels of which were lower in *Clock* mutants compared to wild-type. Notably, several of these genes (i.e., *Per1*, *Per2*, *Per3* and *Cry2*, among others) are targets of the transactivating CLOCK-ARTNL dimer, perhaps revealing reduced activation of expression by the mutated *Clock* gene. Another gene in this cluster is *Clock* itself, as its expression is affected by the genotype at the *Clock* locus.

Diet—The blue cluster was small, containing only two genes: *Tlr4* and *Cxcl1*. Alcohol led to increased expression of both of these genes in the hippocampus.

Genotype*Diet—The pink cluster contained a single gene, *Vip*, and was characterized by a significant genotype by diet interaction. The green cluster was largest in the hippocampus, consisting of 35 genes. The majority of these genes were affected by both genotype and diet. Highest expression levels were typically observed in the alcohol-fed *Clock* mutant mice

compared to all other experimental groups. Expression of the *Cyp2e1* gene, which encodes an enzyme important for the metabolism of alcohol, was influenced by a genotype by alcohol interaction, in addition to significant effects of both genotype and diet alone. This increase in *Cyp2e1* may be related to alcohol-induced changes in other circadian genes, as we have previously shown in the intestine (Forsyth et al., 2011). Among the entire set of 75 genes examined, 20 were selected because of a functional role in the regulation of inflammation. Twelve of these 20 genes (60%) were significantly altered in the hippocampus and grouped together into the green cluster, with increased expression in alcohol-fed *Clock* mutants, suggesting a trend for enrichment of inflammation-related genes in this cluster (odds ratio=2.066, P=0.128, Fisher's exact test). A high proportion of the genes involved in metabolism grouped in the green cluster as well, with 8 of 12 selected metabolism-related genes (67%) being present in this cluster (odds ratio=2.632, P=0.115, Fisher's exact test).

Liver

Fewer genes (22 in total) significantly varied across experimental groups in the liver (Table 3), compared to 56 in the hippocampus (Table 2) and 30 in the proximal colon (Table 4). In the liver, those genes with significant expression changes grouped into five clusters (i.e., cyan, green, blue, orange and pink; Table 3, Figure 2).

Genotype—Genes significantly influenced by genotype grouped into two clusters, with opposing directions of impact. In the green cluster, nine circadian clock genes or CCGs (including the *Pers*, *Tef*, *Dbp* and *Nampt*), as well as one cell-cell junction related gene (*Cdh1*), were decreased in *Clock* mutant mice, while in the orange cluster four other clock genes or CCGs (including *Arntl* and *Cry1*), two inflammation genes (*Pde4b* and *Tlr5*), one metabolism gene (*Pparg*) and one cell-cell junction related gene (*Cldn1*) were increased.

Diet—Both genes in the cyan cluster and the only gene in the pink cluster were significantly affected by alcohol, although the direction of the effect differed: in the pink cluster, alcohol resulted in increased expression of the circadian clock controlled gene *Hnf4a*, whereas it decreased expression of the inflammation-related gene *Hmgb1* and the cancer-related gene *Ccnd1* (cyan cluster).

Genotype*Diet—The blue cluster contained only the oxidative stress-related gene, *Nos2*. Its expression was significantly affected by a genotype by alcohol interaction: expression levels increased in wild-type mice with alcohol, but decreased in alcohol-fed *Clock* mutants.

Proximal Colon

A total of 30 genes were significantly affected by genotype, diet and/or the interaction between the two in the proximal colon (Table 4). These genes grouped into seven clusters (i.e., cyan, orange, green, purple, pink, yellow and blue; Table 4, Figure 3), which revealed distinct effects on gene expression by alcohol and the *Clock* mutation.

Genotype—Genes in the cyan, green and purple clusters contained high proportions of genes affected by genotype, with higher expression levels in *Clock* mutants, regardless of

diet. These three clusters include genes belonging to several functional groups, including metabolism (*Srebf1* and *Prkcd*), cell division/cancer (*Mmp9*), oxidative stress (*Nox1*), brain-gut axis (*Vip*) and cell-cell junction and epithelial permeability (*Cldn1* and *Cdh1*). The blue cluster, which included five genes, showed a significant genotype effect in the opposite direction, with low expression levels in *Clock* mutants regardless of diet. All of the genes in this cluster are under direct control of the CLOCK-ARNTL transcriptional machinery.

Diet—Only four genes were significantly affected by alcohol, including two inflammation-related genes from the orange cluster (*Tnf* and *Cxcl10*), one metabolism gene from the green cluster (*Prkcd*) and one inflammation-related gene from the yellow cluster (*Akp3*).

Genotype*Diet—Several genes in the orange, pink and yellow clusters exhibited complex responses to alcohol and the *Clock* mutation, with a high proportion being significantly affected by a genotype by alcohol interaction. For the pink cluster, higher expression levels tended to be present in the alcohol-fed *Clock* mutants, whereas in the orange and yellow clusters lower levels were observed.

The two genes of the pink cluster exhibited a significant genotype by alcohol interaction. *Hnf1a* belongs to the circadian clock/circadian clock controlled gene category and *Ocln* is an epithelial permeability-related gene. Expression levels were reduced by alcohol in wild-type mice and increased by alcohol in *Clock* mutants.

Four of the six genes in the yellow cluster were significantly affected by a genotype by alcohol interaction. These genes belong to the functional categories of inflammation (*Il17a*, *Cxcl1* and *Ccl2*) and metabolism (*Lep*). Generally, expression was slightly increased by alcohol in the wild-type mice but reduced by alcohol in *Clock* mutants. The two remaining genes in the yellow cluster include an inflammation-related gene that was significantly affected by diet (*Akp3*) and the circadian gene *Clock*, which was significantly affected by genotype, indicating *cis* regulation of expression at the *Clock* locus.

Both genes in the orange cluster, which are inflammation-related genes, were affected by genotype alone and by diet alone. In addition, the gene *Tnf* was affected by a genotype by alcohol interaction: expression levels were not affected by alcohol in wild-type mice, but alcohol led to reduced expression in the *Clock* mutants.

RT-PCR Validation

In order to validate results obtained from the analysis of the array expression data, we performed RT-PCR on *Tlr9* in the hippocampus, *Tlr5* in the liver and *Cdh1* in the proximal colon. In each tissue, expression patterns in different groups observed with RT-PCR generally matched those detected by the array (i.e., the directions of expression changes between groups in the RT-PCR data were consistent with those observed in the analysis of the array data). Although the directions of expression patterns seen in the RT-PCR studies were qualitatively similar to those of the array, the differences between groups failed to reach statistical significance, likely due to the small number of samples available for validation studies (Supporting Information Online).

Discussion

Alterations in expression levels attributed to genotype (i.e., *Clock* mutant vs. wild-type), alcohol (i.e., alcohol vs. dextrose control) and/or a genotype by alcohol interaction were observed in all three tissues, with differing patterns of effect and little overlap observed in each (Figure 4). Of the 75 genes examined, 38 were significantly altered by alcohol in at least one tissue. None of these genes were altered in three tissues simultaneously, and only two were shared across more than one tissue: *Tnf* and *Cxcl10* were both significantly affected in the hippocampus and proximal colon (Figure 4A). A total of 56 genes were significantly altered by the *Clock* mutation in at least one tissue. Eight genes, all of which are involved in the regulation of circadian rhythms or of rhythmic gene expression, were affected in all three tissues and 16 of the remaining 48 genes were affected in two tissues (Figure 4B). Thus, only 43% (24/56) of the genes significantly affected by the mutation were affected in multiple tissues despite the presence of the mutation in all cells and tissues of the body.

Interestingly, there were no genes significantly affected by alcohol in more all three tissues simultaneously and only two were affected in more than one tissue, which suggests that tissues exhibit distinct gene expression response patterns in the presence of alcohol, at least with respect to the genes analyzed in the present study. In contrast, several genes involved or associated with circadian rhythms were significantly affected by the *Clock* mutation in multiple tissues, which supports a molecular circadian clock mechanism that is conserved across tissues, although we do note that the majority of genes examined did exhibit tissue-specificity. Such prominent tissue-specific responses to alcohol and, to a lesser degree, circadian disruption, at least in the case of the *Clock* mutation, may contribute to tissue-specific susceptibility to alcohol toxicity.

In the present study, the hippocampus contained the most genes significantly affected by alcohol, compared to liver and proximal colon, and genes in all categories (circadian rhythms, metabolism, oxidative stress, inflammation, brain-gut axis, cell division/cancer, cell-cell junction and intestinal permeability) were affected. The master circadian clock in the suprachiasmatic nucleus (SCN) of the hypothalamus was not examined because SCN expression responses to alcohol have been studied previously (Filiano et al., 2013) and the primary purpose of the current study was to determine whether expression changes and/or genetic clock disruption in different tissues contribute to alcohol toxicity, not to assess the impact of alcohol on the central circadian timekeeping system. Interestingly, a large proportion of the genes (the green cluster) were significantly affected by both genotype and alcohol in the hippocampus. A high proportion of the genes involved in inflammation and metabolism present on the array were present in this cluster (60% and 67%, respectively), suggesting that the circadian clock and alcohol combine to influence the regulation of inflammatory and metabolic gene expression in the hippocampus.

Given recent evidence linking neuroinflammation to alcohol-induced cognitive dysfunction and brain damage (Kelley and Dantzer, 2011), our results suggest that disruption of circadian rhythms may contribute to the development of cognitive impairment and neurological damage associated with chronic alcoholism through up-regulation of

proinflammatory and metabolic gene expression. Furthermore, the alcohol-metabolizing enzyme *Cyp2e1*, which contributes to the generation of reactive oxygen species (ROS), is significantly affected by a genotype by alcohol interaction, with the highest expression levels observed in *Clock* mutants on the alcohol-containing diet. This implies that the combination of genetic circadian disruption and chronic alcohol exposure interacts to activate *Cyp2e1* expression, which may promote or exacerbate pathological transformation through increased oxidative stress burden.

The fewest number of significantly affected genes were observed in the liver, as compared to the hippocampus and proximal colon. Of these, the majority belonged to the functional group of circadian clock and clock-controlled genes, and most were significantly affected by genotype (Table 3). These results suggest a particularly strong role for the circadian clock in regulating gene expression in the liver, which is known to exhibit robust rhythms of gene expression and physiology (Hughes et al., 2009, Lamia et al., 2008). Indeed, it has been reported that diurnal rhythms of expression of core circadian clock genes and certain metabolic genes are altered in mice exposed to a chronic alcohol consumption protocol (Filiano et al., 2013).

There are several potential reasons that we did not observe greater differential expression in the liver, including: the number and specific functions of genes included in the assay, the duration of the experimental protocol, the possible effects of feeding rhythms on liver gene expression (Vollmers et al., 2009), especially considering the observed changes in diurnal activity rhythms we observed in the present study (Supporting Information), the limitation of gene expression analysis to a single time point and considerations regarding how gene expression changes translate into changes in protein level or function. Interestingly, bone marrow-derived macrophages (BMDMs) from *Clock* mutant mice are less responsive to LPS or TNF and exhibit reduced expression of inflammatory, metabolic and circadian genes (Bellet et al., 2013). The *Clock* mutation may suppress gene expression to a relatively large degree in specific cell types and tissues, perhaps explaining, at least in part, the relative lack of robust, significant changes in expression in the liver observed in this study.

The results of the current study and findings described by Bellet et al. (Bellet et al., 2013), may also explain our previously reported finding that alcohol-fed *Clock* mutant mice failed to exhibit significant liver inflammation or liver cell injury despite increased intestinal permeability and endotoxin levels (Summa et al., 2013). However, these potential limitations seem unlikely to completely explain the findings in the liver because of the significant interaction between alcohol and the circadian clock system in the hippocampus and intestine. To clarify these potential issues, further studies are required to more completely characterize and understand the expression changes in the liver in response to circadian disruption and alcohol.

Within the proximal colon, the majority of genes were significantly affected by genotype. Significantly affected genes belonged to each of functional categories examined (Table 4). The expression of the gene encoding the tight junction protein *Ocln* (occludin) was decreased by alcohol in wild-type mice, which likely contributes to increased alcohol-induced gut leakiness, as we have shown in these mice (Summa et al., 2013). Another

example that may impact intestinal barrier integrity is increased expression of the gene encoding the tight junction protein *Cldn1* (claudin-1), which was increased in *Clock* mutant mice. *Cldn1* encodes a tight junction protein that has paradoxically been associated with increases in intestinal permeability (Han et al., 2003, Prasad et al., 2005, Al-Sadi and Ma, 2007), an effect that appears to be cell-type specific and sensitive to certain environmental exposures. We have previously shown that *Clock* mutant mice have increased intestinal permeability, and these findings suggest that decreased *Ocln* as well as increased *Cldn1* expression may be part of the mechanism underlying this effect. Taken together, our results suggest that interactions between alcohol and the circadian clock are particularly prominent and functionally relevant within the proximal colon, which supports previous *in vitro* and *in vivo* evidence linking the circadian clock and alcohol to the regulation of intestinal barrier integrity (Summa et al., 2013, Swanson et al., 2011).

Our results reveal interesting new details regarding the complex biological effects of alcohol on different tissues; however, we recognize certain limitations inherent in our experimental design. Only a single time point (ZT6) was used for gene expression measurement, thus we were unable to determine whether or not changes in expression exist at other times in the diurnal cycle. However, the mean phase angle of entrainment to a 12:12 LD cycle in *Clock* mutant mice has been shown to be normal with respect to wild-type mice (Vitaterna et al., 1994, Vitaterna et al., 2006), indicating that there is no *a priori* rationale to test for differences in the phase angle of expression rhythms between wild-type and *Clock* mutants. While it has been reported that there is increased ethanol preference in a two-bottle choice paradigm and sensitivity to ethanol-induced sedation in *Clock* mutant mice (Ozburn et al., 2013), suggesting that the *Clock* mutation contributes to alcohol sensitivity and the regulation of alcohol intake. However, those studies used examined mice with a genetic background (BALB/c coisogenic) than ours (C57BL/6J coisogenic). Differences between these strains in responses to alcohol are well-known (Kakihana et al., 1966). We did not observe any differences in alcohol intake between the *Clock* mutants and wild-type mice in our studies (Supporting Online Information). This discrepancy may be due, at least in part, to differences in the genetic background of the mutant strains and/or to the specific diet, source of ethanol and experimental protocols used in each respective study.

Another limitation is that the analysis was restricted to the pre-selected set of 75 genes included in the assay, which may therefore overlook other expression changes. We selected these genes based on their potential importance for: (1) the mechanism(s) of alcohol-induced organ pathology, (2) answering specific questions regarding interactions between alcohol consumption and the circadian clock system, and (3) determining if alcohol and/or circadian-induced effects are universal for all tissues or are specific for a given organ or tissue, which may contribute to tissue-specific susceptibility to alcohol toxicity. Thus, our inclusion of the selected 75 genes in the three different organs was appropriate for the aims and scope of the present study. Finally, gene expression does not always directly translate into changes in protein levels or function. Thus, future studies to evaluate the functional consequences of the observed changes in gene expression will be necessary.

In summary, these results facilitate a maturing understanding of the nature of the molecular changes that occur in response to chronic alcohol consumption and disruption of circadian

rhythms. In particular, they help fill the gap in our knowledge of how alcohol and circadian clock system interactions contribute to adverse pathological consequences in diverse tissues, they may explain in part why different organs have differential susceptibility to alcohol toxicity and they suggest that disruption of circadian rhythms in the context of chronic alcohol use may be a previously unrecognized, and potentially modifiable, risk factor for the development or progression of alcohol-related pathologies. While these studies utilized a genetic model of circadian disruption, we recently observed similar alcohol-induced physiological responses in the intestine of both *Clock* mutant and phase-shifted mice (Summa et al., 2013), the latter likely being a more accurate model for humans with disrupted circadian rhythms, suggesting that responses at the level of gene expression may be similar. Strategies designed to limit circadian misalignment or that target the circadian clock to improve organization of circadian rhythms may provide useful approaches for the prevention and/or mitigation of alcohol-related pathologies. This study provides strong scientific rationale to study the efficacy of circadian clock-directed interventions, such as chronotherapy, for the prevention and/or treatment of alcohol-related pathologies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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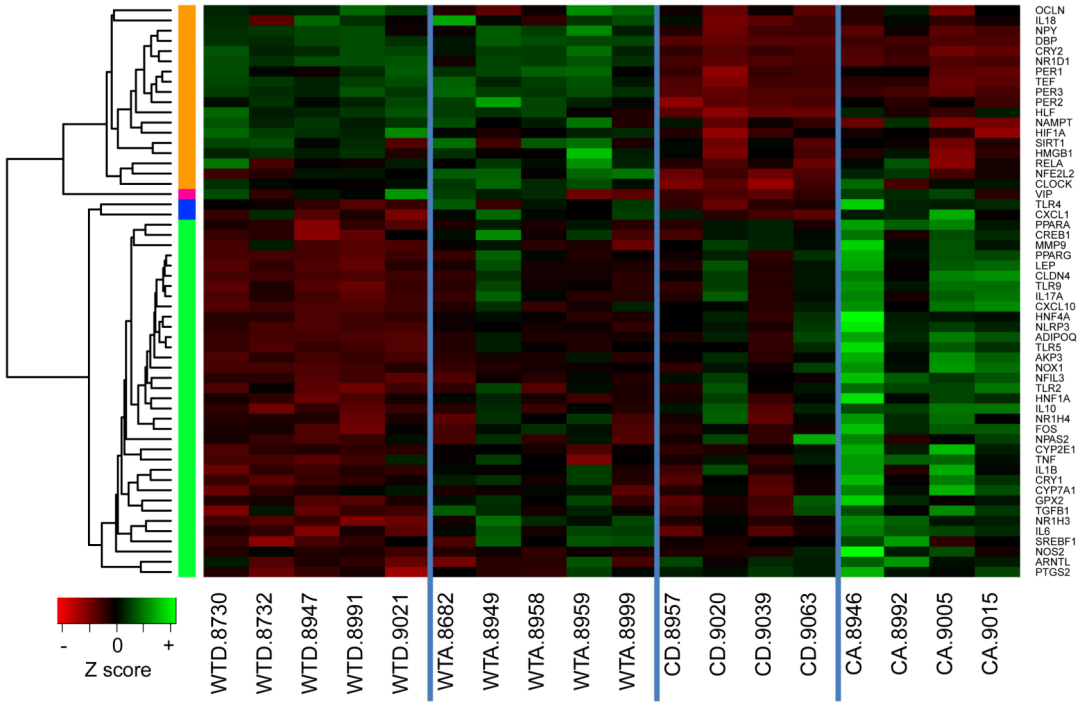

Figure 1. Hippocampal gene expression levels in wild-type (WT) and *Clock* mutant (C) mice on either an alcohol (A) or dextrose-containing control (D) diet

Expression levels of genes exhibiting significant changes in the hippocampus for at least one term (genotype, alcohol or genotype by alcohol interaction) were normalized as Z scores and plotted as a heatmap. Genes with similar expression patterns were grouped into clusters, depicted by colors on the left side of the graph. The orange cluster reflects groups of genes influenced by genotype. The blue cluster includes genes affected by diet. The pink cluster contains a single gene, *Vip*, that is characterized by a genotype by diet interaction. The green cluster contains genes affected by genotype as well as alcohol. Cluster color was assigned independently and randomly in each tissue, thus there is no implied association between clusters of the same color in different tissues. Gene symbols are listed on the right side. There were four experimental groups: wild-type dextrose (WTD), wild-type alcohol (WTA), *Clock* mutant dextrose (CD) and *Clock* mutant alcohol (CA). Each column represents an individual mouse, denoted by the ID number listed after the experimental group designation. Within the heatmap, green indicates increased expression and red indicates reduced expression, with color intensity reflecting the magnitude of the expression change.

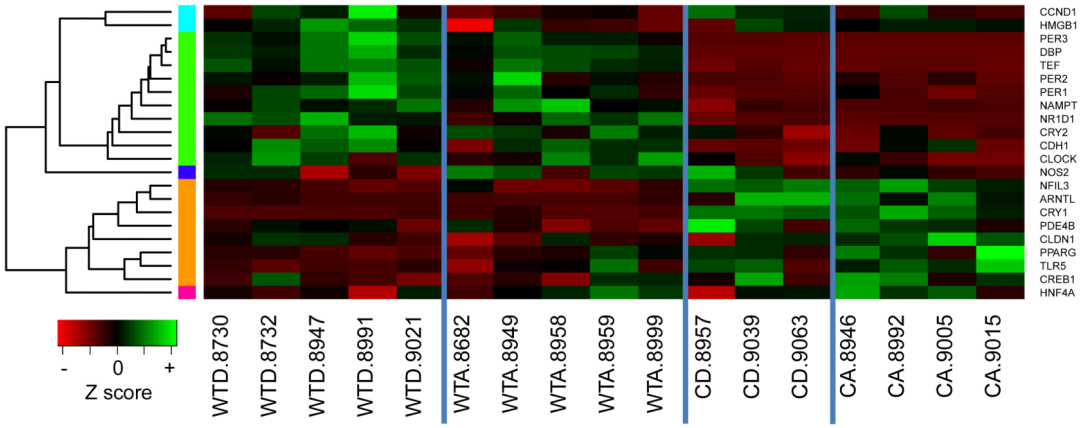

Figure 2. Liver gene expression levels in wild-type (WT) and *Clock* mutant (C) mice on either an alcohol (A) or dextrose-containing control (D) diet

Expression levels of genes exhibiting significant changes for at least one term (genotype, alcohol or genotype by alcohol interaction) in the liver were normalized as Z scores and plotted as a heatmap. Genes with similar expression patterns were grouped into clusters, depicted by colors on the left side of the graph. The green and orange clusters include genes influenced by genotype. The pink and cyan clusters include genes affected by diet. The blue cluster contains a single gene influenced by a genotype by diet interaction (*Nos2*). Cluster color was assigned independently and randomly in each tissue, thus there is no implied association between clusters of the same color in different tissues. Gene symbols are listed on the right side. There were four experimental groups: wild-type dextrose (WTD), wild-type alcohol (WTA), *Clock* mutant dextrose (CD) and *Clock* mutant alcohol (CA). Each column represents an individual mouse, denoted by the ID number listed after the experimental group designation. Within the heatmap, green indicates increased expression and red indicates reduced expression, with color intensity reflecting the magnitude of the expression change.

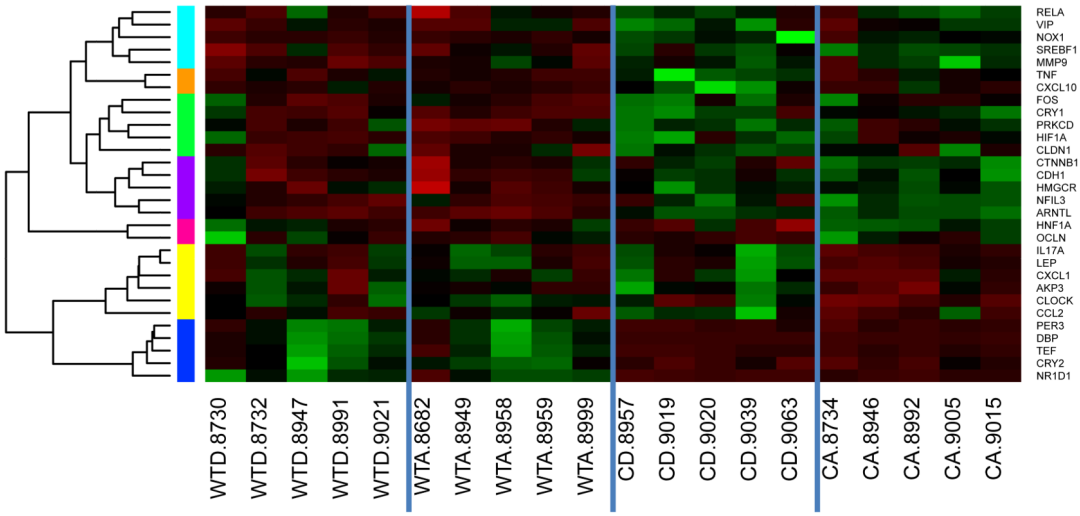

Figure 3. Proximal colon expression levels in wild-type (WT) and *Clock* mutant (C) mice on either an alcohol (A) or dextrose-containing control (D) diet

Expression levels of genes exhibiting significant changes for at least one term (genotype, alcohol or genotype by alcohol interaction) in the proximal colon were normalized as Z scores and plotted as a heatmap. Genes with similar expression patterns were grouped into clusters, depicted by colors on the left side of the graph. The cyan, blue, green and purple clusters contain genes influenced by genotype. The orange cluster includes genes significantly affected by diet. The pink, orange and yellow clusters contain genes impacted by a genotype by alcohol interaction. Cluster color was assigned independently and randomly in each tissue, thus there is no implied association between clusters of the same color in different tissues. Gene symbols are listed on the right side. There were four experimental groups: wild-type dextrose (WTD), wild-type alcohol (WTA), *Clock* mutant dextrose (CD) and *Clock* mutant alcohol (CA). Each column represents an individual mouse, denoted by the ID number listed after the experimental group designation. Within the heatmap, green indicates increased expression and red indicates reduced expression, with color intensity reflecting the magnitude of the expression change.

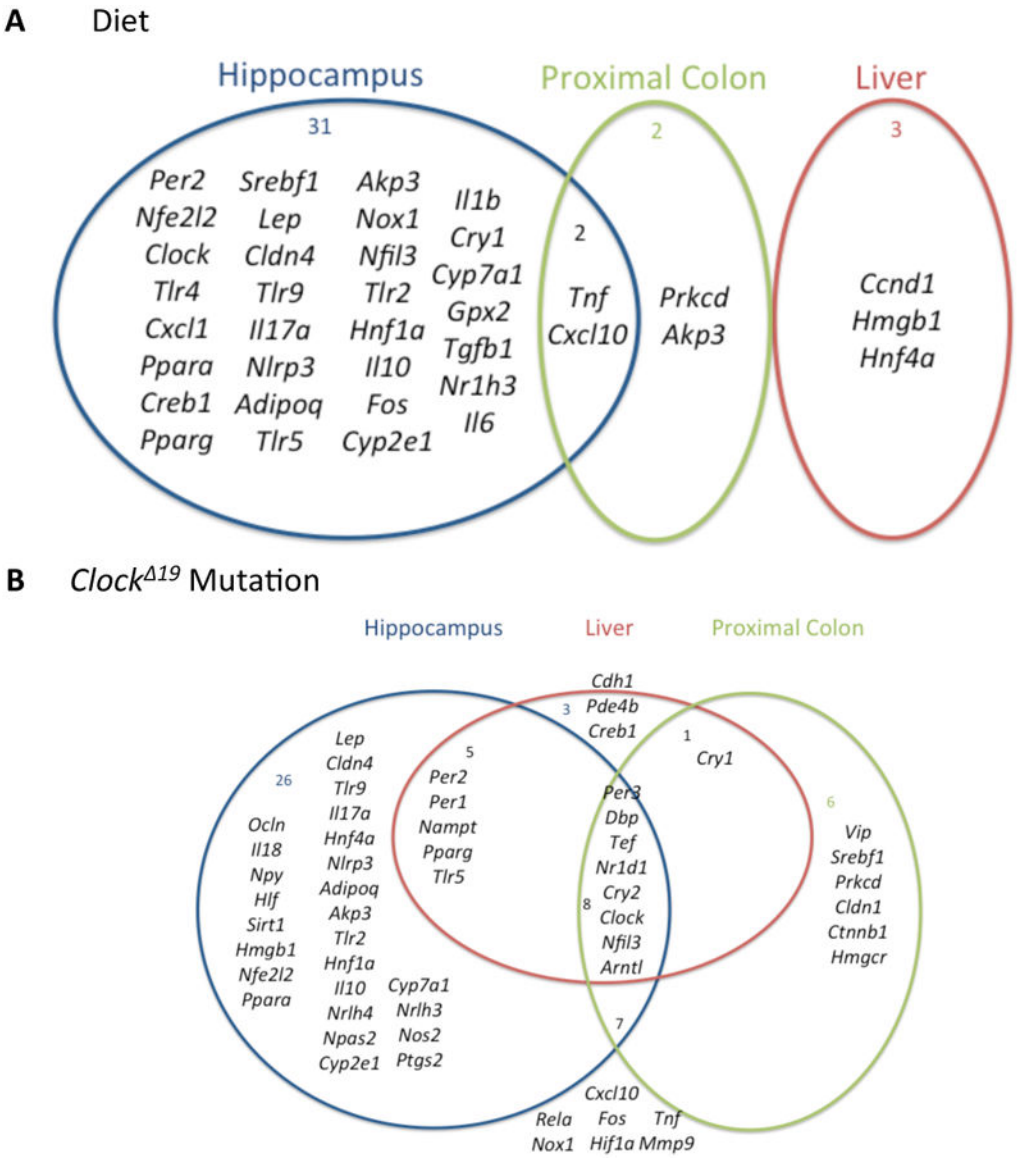

Figure 4. Alcohol (A) and the circadian *Clock*$^{\Delta 19}$ mutation (B) exert tissue-specific effects on gene expression in the mouse hippocampus, liver and proximal colon

(A) Venn diagram listing genes significantly affected by alcohol in the hippocampus (blue circle), liver (red circle) and proximal colon (green circle) in this study. There was very little overlap of significantly affected genes across tissues. **(B)** Venn diagram listing genes significantly affected by the circadian *Clock*$^{\Delta 19}$ mutation in the hippocampus (blue circle), liver (red circle) and proximal colon (green circle) in this study.

Table 1

Genes analyzed for expression levels using a custom-designed Affymetrix 80Plex bead chip

The selected genes were chosen for analysis because of their classification in one of the following functional categories: circadian clock and clock-controlled genes, inflammation, metabolism, oxidative stress, cell-junction and epithelial permeability, brain-gut axis and cancer.

Gene Symbol	Gene Name	RefSeq No.	Functional Category
Arntl	aryl hydrocarbon receptor nuclear translocator-like	NM_007489	clock and clock-controlled genes
Clock	circadian locomotor output cycles kaput	NM_007715	clock and clock-controlled genes
Creb1	cAMP responsive element binding protein 1	NM_133828	clock and clock-controlled genes
Cry1	cryptochrome 1 (photolyase-like)	NM_007771	clock and clock-controlled genes
Cry2	cryptochrome 2 (photolyase-like)	NM_009963	clock and clock-controlled genes
Csnk1e	casein kinase 1, epsilon	NM_013767	clock and clock-controlled genes
Dbp	D site albumin promoter binding protein	NM_016974	clock and clock-controlled genes
Hlf	hepatic leukemia factor	NM_172563	clock and clock-controlled genes
Hnf1a	HNF1 homeobox A	NM_009327	clock and clock-controlled genes
Hnf4a	hepatic nuclear factor 4, alpha	NM_008261	clock and clock-controlled genes
Nampt	nicotinamide phosphoribosyltransferase	NM_021524	clock and clock-controlled genes
Nfil3	nuclear factor, interleukin 3, regulated	NM_017373	clock and clock-controlled genes
Npas2	neuronal PAS domain protein 2	NM_008719	clock and clock-controlled genes
Nr1d1	nuclear receptor subfamily 1, group D, member 1	NM_145434	clock and clock-controlled genes
Per1	period 1	NM_011065	clock and clock-controlled genes
Per2	period 2	NM_011066	clock and clock-controlled genes
Per3	period 3	NM_011067	clock and clock-controlled genes
Prkab1	protein kinase, AMP-activated, beta 1 non-catalytic subunit	NM_031869	clock and clock-controlled genes
Rora	RAR-related orphan receptor alpha	NM_013646	clock and clock-controlled genes
Sirt1	sirtuin 1	NM_019812	clock and clock-controlled genes
Tef	thyrotrophic embryonic factor	NM_017376	clock and clock-controlled genes
Akp3	alkaline phosphatase 3, intestine, not Mn requiring	NM_007432	inflammation
Ccl2	chemokine (C-C motif) ligand 2	NM_011333	inflammation
Cxcl1	chemokine (C-X-C motif) ligand 1	NM_008176	inflammation
Cxcl10	chemokine (C-X-C motif) ligand 10	NM_021274	inflammation
Hmgb1	high mobility group box 1	NM_010439	inflammation
Il10	interleukin 10	NM_010548	inflammation
Il17a	interleukin 17A	NM_010552	inflammation
Il18	interleukin 18	NM_008360	inflammation
Il1b	interleukin 1 beta	NM_008361	inflammation
Il6	interleukin 6	NM_031168	inflammation
Lbp	lipopolysaccharide binding protein	NM_008489	inflammation
Nlrp3	NLR family, pyrin domain containing 3	NM_145827	inflammation
Pde4b	phosphodiesterase 4B, cAMP specific	NM_019840	inflammation
Ptgs2	prostaglandin-endoperoxide synthase 2	NM_011198	inflammation

Gene Symbol	Gene Name	RefSeq No.	Functional Category
Rela	v-rel reticuloendotheliosis viral oncogene homolog A (avian)	NM_009045	inflammation
Tlr2	toll-like receptor 2	NM_011905	inflammation
Tlr4	toll-like receptor 4	NM_021297	inflammation
Tlr5	toll-like receptor 5	NM_016928	inflammation
Tlr9	toll-like receptor 9	NM_031178	inflammation
Tnf	tumor necrosis factor	NM_013693	inflammation
Adipoq	adiponectin, C1Q and collagen domain containing	NM_009605	metabolism
Ccrn4l	CCR4 carbon catabolite repression 4-like (S. cerevisiae)	NM_009834	metabolism
Cyp7a1	cytochrome P450, family 7, subfamily a, polypeptide 1	NM_007824	metabolism
Hmgcr	3-hydroxy-3-methylglutaryl-Coenzyme A reductase	NM_008255	metabolism
Lep	leptin	NM_008493	metabolism
Nr1h3	nuclear receptor subfamily 1, group H, member 3	NM_013839	metabolism
Nr1h4	nuclear receptor subfamily 1, group H, member 4	NM_009108	metabolism
Ppara	peroxisome proliferator activated receptor alpha	NM_011144	metabolism
Pparg	peroxisome proliferator-activated receptor gamma	NM_011146	metabolism
Ppargc1a	peroxisome proliferative activated receptor, gamma, coactivator 1 alpha	NM_008904	metabolism
Prkcd	protein kinase C, delta	NM_011103	metabolism
Srebf1	sterol regulatory element binding transcription factor 1	NM_011480	metabolism
Cyp2e1	cytochrome P450, family 2, subfamily e, polypeptide 1	NM_021282	oxidative stress
Gpx2	glutathione peroxidase 2	NM_030677	oxidative stress
Nfe2l2	nuclear factor, erythroid derived 2, like 2	NM_010902	oxidative stress
Nos1	nitric oxide synthase 1, neuronal	NM_008712	oxidative stress
Nos2	nitric oxide synthase 2, inducible	NM_010927	oxidative stress
Nox1	NADPH oxidase 1	NM_172203	oxidative stress
Ucp2	uncoupling protein 2 (mitochondrial, proton carrier)	NM_011671	oxidative stress
Cdh1	cadherin 1, type 1, E-cadherin (epithelial)	NM_009864	cell-junction and epithelial permeability
Cldn1	claudin 1	NM_016674	cell-junction and epithelial permeability
Cldn2	claudin 2	NM_016675	cell-junction and epithelial permeability
Cldn4	claudin 4	NM_009903	cell-junction and epithelial permeability
Ocln	occludin	NM_008756	cell-junction and epithelial permeability
Tjp1	tight junction protein 1	NM_009386	cell-junction and epithelial permeability
Fos	FBJ osteosarcoma oncogene	NM_010234	brain-gut axis
Gfap	glial fibrillary acidic protein	NM_010277	brain-gut axis
Npy	neuropeptide Y	NM_023456	brain-gut axis
Vip	vasoactive intestinal polypeptide	NM_011702	brain-gut axis
Ccnd1	cyclin D1	NM_007631	cancer
Ctnnb1	catenin (cadherin associated protein), beta 1	NM_007614	cancer
Hif1a	hypoxia inducible factor 1, alpha subunit	NM_010431	cancer
Mmp9	matrix metallopeptidase 9	NM_013599	cancer

Gene Symbol	Gene Name	RefSeq No.	Functional Category
Tgfb1	transforming growth factor, beta 1	NM_011577	cancer

Table 2

Genes that varied significantly across experimental groups in the hippocampus

Genes are grouped into clusters, as shown in Figure 1.

		2-way ANOVA			
Gene Symbol	**Functional Category**	**Genotype**	**Diet**	**G × D interaction**	**Clusters**
Ocln	cell-junction and epithelial permeability	$F_{1,14}$ = 6.32, P = 0.0248	n.s.	n.s.	orange
Il18	inflammation	$F_{1,14}$ = 6.52, P = 0.023	n.s.	n.s.	orange
Npy	brain-gut axis	$F_{1,14}$ = 36.01, P = 3.25E-05	n.s.	n.s.	orange
Dbp	clock and clock-controlled genes	$F_{1,14}$ = 311.04, P = 5.87E-11	n.s.	n.s.	orange
Cry2	clock and clock-controlled genes	$F_{1,14}$ = 125.28, P = 2.27E-08	n.s.	n.s.	orange
Nr1d1	clock and clock-controlled genes	$F_{1,14}$ = 141.13, P = 1.06E-08	n.s.	n.s.	orange
Per1	clock and clock-controlled genes	$F_{1,14}$ = 30.34, P = 7.71E-05	n.s.	n.s.	orange
Tef	clock and clock-controlled genes	$F_{1,14}$ = 100.31, P = 9.17E-08	n.s.	n.s.	orange
Per3	clock and clock-controlled genes	$F_{1,14}$ = 266.57, P = 1.65E-10	n.s.	n.s.	orange
Per2	clock and clock-controlled genes	$F_{1,14}$ = 36.92, P = 2.86E-05	$F_{1,14}$ = 6.75, P = 0.021	n.s.	orange
Hlf	clock and clock-controlled genes	$F_{1,14}$ = 34.36, P = 4.13E-05	n.s.	$F_{1,14}$ = 15.23, P = 0.0016	orange
Nampt	clock and clock-controlled genes	$F_{1,14}$ = 13.72, P = 0.00236	n.s.	n.s.	orange
Hif1a	cancer	$F_{1,14}$ = 18.82, P = 0.000681	n.s.	n.s.	orange
Sirt1	clock and clock-controlled genes	$F_{1,14}$ = 6.1, P = 0.027	n.s.	n.s.	orange
Hmgb1	inflammation	$F_{1,14}$ = 8.09, P = 0.013	n.s.	n.s.	orange
Rela	inflammation	$F_{1,14}$ = 4.86, P = 0.0448	n.s.	n.s.	orange
Nfe2l2	oxidative stress	$F_{1,14}$ = 13.75, P = 0.00234	$F_{1,14}$ = 18.66, P = 0.000707	n.s.	orange
Clock	clock and clock-controlled genes	$F_{1,14}$ = 15.64, P = 0.00144	$F_{1,14}$ = 16.02, P = 0.00131	n.s.	orange
Vip	brain-gut axis	n.s.	n.s.	$F_{1,14}$ = 6.41, P = 0.0239	pink
Tlr4	inflammation	n.s.	$F_{1,14}$ = 13.37, P = 0.00259	n.s.	blue
Cxcl1	inflammation	n.s.	$F_{1,14}$ = 10.94, P = 0.00519	n.s.	blue
Ppara	metabolism	$F_{1,14}$ = 17.61, P = 0.000897	$F_{1,14}$ = 14.92, P = 0.00172	n.s.	green
Creb1	clock and clock-controlled genes	n.s.	$F_{1,14}$ = 6.69, P = 0.0216	n.s.	green
Mmp9	cancer	$F_{1,14}$ = 12.86, P = 0.00298	n.s.	n.s.	green

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		2-way ANOVA			
Gene Symbol	**Functional Category**	**Genotype**	**Diet**	**G × D interaction**	**Clusters**
Pparg	metabolism	$F_{1,14}$ = 12.19, P = 0.0036	$F_{1,14}$ = 10.58, P = 0.00578	n.s.	green
Lep	metabolism	$F_{1,14}$ = 12.75, P = 0.00307	$F_{1,14}$ = 8.95, P = 0.00971	n.s.	green
Cldn4	cell-junction and epithelial permeability	$F_{1,14}$ = 22.34, P = 0.000325	$F_{1,14}$ = 11.38, P = 0.00455	n.s.	green
Tlr9	inflammation	$F_{1,14}$ = 16.83, P = 0.00108	$F_{1,14}$ = 13.97, P = 0.00221	n.s.	green
Il17a	inflammation	$F_{1,14}$ = 9.4, P = 0.00837	$F_{1,14}$ = 6.36, P = 0.0244	n.s.	green
Cxcl10	inflammation	$F_{1,14}$ = 19.39, P = 0.000601	$F_{1,14}$ = 16.4, P = 0.00119	n.s.	green
Hnf4a	clock and clock-controlled genes	$F_{1,14}$ = 7.98, P = 0.0135	n.s.	n.s.	green
Nlrp3	inflammation	$F_{1,14}$ = 16.19, P = 0.00126	$F_{1,14}$ = 6.77, P = 0.0209	n.s.	green
Adipoq	metabolism	$F_{1,14}$ = 29.65, P = 8.63E-05	$F_{1,14}$ = 17.33, P = 0.000956	n.s.	green
Tlr5	inflammation	$F_{1,14}$ = 13.7, P = 0.00237	$F_{1,14}$ = 10.68, P = 0.00561	n.s.	green
Akp3	inflammation	$F_{1,14}$ = 24.75, P = 0.000204	$F_{1,14}$ = 12.93, P = 0.00293	n.s.	green
Nox1	oxidative stress	$F_{1,14}$ = 29.38, P = 9.02E-05	$F_{1,14}$ = 12.36, P = 0.00343	n.s.	green
Nfil3	clock and clock-controlled genes	$F_{1,14}$ = 36.41, P = 3.07E-05	$F_{1,14}$ = 10.29, P = 0.00632	n.s.	green
Tlr2	inflammation	$F_{1,14}$ = 18.58, P = 0.000718	$F_{1,14}$ = 8.78, P = 0.0103	n.s.	green
Hnf1a	clock and clock-controlled genes	$F_{1,14}$ = 8.04, P = 0.0132	$F_{1,14}$ = 5.1, P = 0.0403	n.s.	green
Il10	inflammation	$F_{1,14}$ = 21.07, P = 0.00042	$F_{1,14}$ = 9.42, P = 0.00832	n.s.	green
Nr1h4	metabolism	$F_{1,14}$ = 4.78, P = 0.0463	n.s.	n.s.	green
Fos	brain-gut axis	$F_{1,14}$ = 8.91, P = 0.00984	$F_{1,14}$ = 10.94, P = 0.00518	n.s.	green
Npas2	clock and clock-controlled genes	$F_{1,14}$ = 5.07, P = 0.0409	n.s.	n.s.	green
Cyp2e1	oxidative stress	$F_{1,14}$ = 12.16, P = 0.00362	$F_{1,14}$ = 9.26, P = 0.00877	$F_{1,14}$ = 4.66, P = 0.0487	green
Tnf	inflammation	$F_{1,14}$ = 8.02, P = 0.0133	$F_{1,14}$ = 5.85, P = 0.0298	n.s.	green
Il1b	inflammation	n.s.	$F_{1,14}$ = 7.2, P = 0.0179	n.s.	green
Cry1	clock and clock-controlled genes	n.s.	$F_{1,14}$ = 16.52, P = 0.00116	n.s.	green
Cyp7a1	metabolism	$F_{1,14}$ = 5.84, P = 0.0299	$F_{1,14}$ = 7.19, P = 0.0179	$F_{1,14}$ = 6.71, P = 0.0214	green
Gpx2	oxidative stress	n.s.	$F_{1,14}$ = 5.66, P = 0.0321	n.s.	green
Tgfb1	cancer	n.s.	$F_{1,14}$ = 11.5, P = 0.00439	n.s.	green
Nr1h3	metabolism	$F_{1,14}$ = 6.01, P = 0.028	$F_{1,14}$ = 32.14, P = 5.79E-05	n.s.	green

		2-way ANOVA			
Gene Symbol	**Functional Category**	**Genotype**	**Diet**	**G × D interaction**	**Clusters**
Il6	inflammation	n.s.	$F_{1,14}$ = 33.53, P = 4.68E-05	n.s.	green
Srebf1	metabolism	n.s.	$F_{1,14}$ = 7.03, P = 0.019	n.s.	green
Nos2	oxidative stress	$F_{1,14}$ = 4.9, P = 0.0439	n.s.	n.s.	green
Arntl	clock and clock-controlled genes	$F_{1,14}$ = 11.51, P = 0.00437	n.s.	n.s.	green
Ptgs2	inflammation	$F_{1,14}$ = 12.17, P = 0.00362	$F_{1,14}$ = 5.06, P = 0.0412	n.s.	green

Table 3

Genes that varied significantly across experimental groups in the liver

Genes are grouped into clusters, as shown in Figure 2.

		2-way ANOVA			
Gene Symbol	**Functional Category**	**Genotype**	**Diet**	**G × D interaction**	**Clusters**
Ccnd1	cancer	n.s.	$F_{1,13}$ = 6.16, P = 0.0275	n.s.	cyan
Hmgb1	inflammation	n.s.	$F_{1,13}$ = 4.72, P = 0.0488	n.s.	cyan
Per3	clock and clock-controlled genes	$F_{1,13}$ = 41.56, P = 2.18E-05	n.s.	n.s.	green
Dbp	clock and clock-controlled genes	$F_{1,13}$ = 76.41, P = 8.37E-07	n.s.	n.s.	green
Tef	clock and clock-controlled genes	$F_{1,13}$ = 80.18, P = 6.38E-07	n.s.	n.s.	green
Per2	clock and clock-controlled genes	$F_{1,13}$ = 10.5, P = 0.00644	n.s.	n.s.	green
Per1	clock and clock-controlled genes	$F_{1,13}$ = 18.04, P = 0.000953	n.s.	n.s.	green
Nampt	clock and clock-controlled genes	$F_{1,13}$ = 17.32, P = 0.00112	n.s.	n.s.	green
Nr1d1	clock and clock-controlled genes	$F_{1,13}$ = 27.48, P = 0.000159	n.s.	n.s.	green
Cry2	clock and clock-controlled genes	$F_{1,13}$ = 8.03, P = 0.0141	n.s.	n.s.	green
Cdh1	cell-junction and epithelial permeability	$F_{1,13}$ = 9.54, P = 0.00862	n.s.	n.s.	green
Clock	clock and clock-controlled genes	$F_{1,13}$ = 10.05, P = 0.00738	n.s.	n.s.	green
Nos2	oxidative stress	n.s.	n.s.	$F_{1,13}$ = 5.76, P = 0.0321	blue
Nfil3	clock and clock-controlled genes	$F_{1,13}$ = 62.1, P = 2.64E-06	n.s.	n.s.	orange
Arntl	clock and clock-controlled genes	$F_{1,13}$ = 25.47, P = 0.000224	n.s.	n.s.	orange
Cry1	clock and clock-controlled genes	$F_{1,13}$ = 127.62, P = 4.3E-08	n.s.	n.s.	orange
Pde4b	inflammation	$F_{1,13}$ = 6.57, P = 0.0236	n.s.	n.s.	orange
Cldn1	cell-junction and epithelial permeability	n.s.	n.s.	$F_{1,13}$ = 6.02, P = 0.029	orange
Pparg	metabolism	$F_{1,13}$ = 7.91, P = 0.0147	n.s.	n.s.	orange
Tlr5	inflammation	$F_{1,13}$ = 8.24, P = 0.0131	n.s.	n.s.	orange
Creb1	clock and clock-controlled genes	$F_{1,13}$ = 4.89, P = 0.0456	n.s.	n.s.	orange
Hnf4a	clock and clock-controlled genes	n.s.	$F_{1,13}$ = 5.17, P = 0.0406	n.s.	pink

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Table 4

Genes that varied significantly across experimental groups in the proximal colon

Genes are grouped into clusters, as shown in Figure 3.

		2-way ANOVA			
Gene Symbol	**Functional Category**	**Genotype**	**Diet**	**G × D interaction**	**Clusters**
Rela	inflammation	$F_{1,16}$ = 7.01, P = 0.0176	n.s.	n.s.	cyan
Vip	brain-gut axis	$F_{1,16}$ = 6.9, P = 0.0183	n.s.	n.s.	cyan
Nox1	oxidative stress	$F_{1,16}$ = 12.18, P = 0.00303	n.s.	n.s.	cyan
Srebf1	metabolism	$F_{1,16}$ = 20.28, P = 0.000361	n.s.	n.s.	cyan
Mmp9	cancer	$F_{1,16}$ = 8.8, P = 0.00909	n.s.	n.s.	cyan
Tnf	inflammation	$F_{1,16}$ = 9.83, P = 0.00639	$F_{1,16}$ = 7.84, P = 0.0128	$F_{1,16}$ = 4.73, P = 0.045	orange
Cxcl10	inflammation	$F_{1,16}$ = 5.95, P = 0.0268	$F_{1,16}$ = 5.61, P = 0.0308	n.s.	orange
Fos	brain-gut axis	$F_{1,16}$ = 4.53, P = 0.0493	n.s.	n.s.	green
Cry1	clock and clock-controlled genes	$F_{1,16}$ = 16.25, P = 0.000967	n.s.	n.s.	green
Prkcd	metabolism	$F_{1,16}$ = 9.03, P = 0.00838	$F_{1,16}$ = 4.87, P = 0.0423	n.s.	green
Hif1a	cancer	$F_{1,16}$ = 7.75, P = 0.0133	n.s.	n.s.	green
Cldn1	cell-junction and epithelial permeability	$F_{1,16}$ = 6.26, P = 0.0235	n.s.	n.s.	green
Ctnnb1	cancer	$F_{1,16}$ = 6.03, P = 0.0259	n.s.	$F_{1,16}$ = 4.84, P = 0.0429	purple
Cdh1	cell-junction and epithelial permeability	$F_{1,16}$ = 12.03, P = 0.00316	n.s.	n.s.	purple
Hmgcr	metabolism	$F_{1,16}$ = 11.48, P = 0.00375	n.s.	n.s.	purple
Nfil3	clock and clock-controlled genes	$F_{1,16}$ = 16.76, P = 0.000848	n.s.	n.s.	purple
Arntl	clock and clock-controlled genes	$F_{1,16}$ = 110.22, P = 1.39E-08	n.s.	n.s.	purple
Hnf1a	clock and clock-controlled genes	n.s.	n.s.	$F_{1,16}$ = 12.02, P = 0.00318	pink
Ocln	cell-junction and epithelial permeability	n.s.	n.s.	$F_{1,16}$ = 5.51, P = 0.0321	pink
Il17a	inflammation	n.s.	n.s.	$F_{1,16}$ = 5.53, P = 0.0318	yellow
Lep	metabolism	n.s.	n.s.	$F_{1,16}$ = 5.22, P = 0.0363	yellow
Cxcl1	inflammation	n.s.	n.s.	$F_{1,16}$ = 4.53, P = 0.0492	yellow
Akp3	inflammation	n.s.	$F_{1,16}$ = 8.79, P = 0.00912	n.s.	yellow
Clock	clock and clock-controlled genes	$F_{1,16}$ = 9.37, P = 0.00746	n.s.	n.s.	yellow

		2-way ANOVA			
Gene Symbol	**Functional Category**	**Genotype**	**Diet**	**G × D interaction**	**Clusters**
Ccl2	Inflammation	n.s.	n.s.	$F_{1,16}$ = 4.6, P = 0.0477	yellow
Per3	clock and clock-controlled genes	$F_{1,16}$ = 17.39, P = 0.000722	n.s.	n.s.	blue
Dbp	clock and clock-controlled genes	$F_{1,16}$ = 23.98, P = 0.000161	n.s.	n.s.	blue
Tef	clock and clock-controlled genes	$F_{1,16}$ = 17.54, P = 0.000696	n.s.	n.s.	blue
Cry2	clock and clock-controlled genes	$F_{1,16}$ = 18.4, P = 0.000562	n.s.	n.s.	blue
Nr1d1	clock and clock-controlled genes	$F_{1,16}$ = 28.07, P = 7.22E-05	n.s.	n.s.	blue