Published in final edited form as:

Toxicol Appl Pharmacol. 2018 January 15; 339: 110–120. doi:10.1016/j.taap.2017.12.005.

Valproic acid disrupts the oscillatory expression of core circadian rhythm transcription factors

Chanel A. Griggs, Scott W. Malm, Rosa Jaime-Frias, and Catharine L. Smith, PhD¹

Department of Pharmacology and Toxicology, College of Pharmacy, University of Arizona, Tucson, AZ, 85721, United States

Abstract

Valproic acid (VPA) is a well-established therapeutic used in treatment of seizure and mood disorders as well as migraines and a known hepatotoxicant. About 50% of VPA users experience metabolic disruptions, including weight gain, hyperlipidemia, and hyperinsulinemia, among others. Several of these metabolic abnormalities are similar to the effects of circadian rhythm disruption. In the current study, we examine the effect of VPA exposure on the expression of core circadian transcription factors that drive the circadian clock via a transcription-translation feedback loop. In cells with an unsynchronized clock, VPA simultaneously upregulated the expression of genes encoding core circadian transcription factors that regulate the positive and negative limbs of the feedback loop. Using low dose glucocorticoid, we synchronized cultured fibroblast cells to a circadian oscillatory pattern. Whether VPA was added at the time of synchronization or 12 h later at CT12, we found that VPA disrupted the oscillatory expression of multiple genes encoding essential transcription factors that regulate circadian rhythm. Therefore, we conclude that VPA has a potent effect on the circadian rhythm transcription-translation feedback loop that may be linked to negative VPA side effects in humans. Furthermore, our study suggests potential chronopharmacology implications of VPA usage.

Keywords

circadian rhythm; transcription; histone deacetylase inhibitor; glucocorticoids

INTRODUCTION

Most organisms operate on a roughly 24-hour light/dark cycle, known as circadian rhythm. The regulation of circadian rhythm involves a tightly-controlled hierarchy system orchestrating rhythmicity in the brain and peripheral tissues (Farnell *et al.*, 2011; Albrecht, 2012). Both clinical and animal studies have established that disruption of circadian rhythm

¹To whom correspondence should be addressed at Department of Pharmacology and Toxicology, University of Arizona, College of Pharmacy, 1703 E. Mabel St., Tucson AZ 85721-0207; 520-626-8349; csmith@pharmacy.arizona.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conflict of Interest Statement: The authors have nothing to disclose.

is associated with a variety of pathological states that include perturbations of metabolism, immune function, sleep, and fertility, among others (Sen *et al.*, 2016; Castanon-Cervantes *et al.*, 2010; McHill *et al.*, 2017; Morikawa *et al.*, 2005; Karlsson *et al.*, 2001). Circadian misalignment with environmental rhythms can arise from modern-day lifestyles that involve long-distance jet travel, exposure to nighttime light, erratic meal schedules, and shift work. In addition, xenobiotics, including environmental toxicants (Jimenez-Ortega *et al.*, 2012; Garrett *et al.*, 2006; Xu *et al.*, 2010) and drugs (Li *et al.*, 2007; Li *et al.*, 2002; Johansson *et al.*, 2011; Koyanagi *et al.*, 2002; Iurisci *et al.*, 2009; Terazono *et al.*, 2008; Ortiz-Tudela *et al.*, 2014; Osland *et al.*, 2011), have been shown to cause disruptions to circadian rhythm. A recent study showed that more than 50% of all drugs approved for clinical use target genes or proteins that show rhythmic expression in mice (Zhang *et al.*, 2014). As human exposure to xenobiotics continues to increase, it is imperative to develop a comprehensive understanding of their impact on circadian rhythms as well as how circadian rhythm affects their metabolism, potency, and toxicity.

Mammalian circadian timing is driven by the master clock, located in the suprachiasmatic nucleus (SCN) of the hypothalamus, which generates signals to synchronize peripheral clocks in other tissues [reviewed in (Albrecht, 2012)]. These tissues express autoregulatory clock proteins that generate circadian rhythmicity by transcription-translation negative feedback loops (Shearman *et al.*, 2000). Core components of these feedback loops include Clock and Bmal1, members of the positive limb, and negative limb members Period1, Period2, Period3 (Per1, Per2 and Per3), Cryptochrome1 and Cryptochrome2 (Cry1 and Cry2), and Rev-erba and β . Clock and Bmal1 heterodimerize and activate transcription by binding to E box sequences in target gene promoters, including those of genes encoding the negative limb components. Per and Cry proteins accumulate and physically interact with the Clock/Bmal1 heterodimer at E boxes to block activated transcription (Shearman *et al.*, 2000; Zheng *et al.*, 1999; Kume *et al.*, 1999). In addition, Rev-erbs (α , β) and RORs (α , β , γ) control the cyclic expression of the *Bmal1* gene, with RORs inducing expression and Rev-erbs suppressing it (Partch *et al.*, 2014; Sato *et al.*, 2004; Preitner *et al.*, 2002). The outcome of this process is the oscillatory expression and subsequent activity of the circadian rhythm transcription factors in both the brain and peripheral tissues (Jin *et al.*, 1999). A study to comprehensively catalog circadian gene expression in mice showed that this regulatory loop has been found to confer rhythmic expression on about 43% of all genes in at least one tissue type (Zhang *et al.*, 2014).

The equilibrium between lysine acetyltransferases (KATs, also known as histone acetyltransferases or HATs) and lysine deacetylases (KDACs, also known as histone deacetylases or HDACs) is hypothesized to be critical for proper circadian gene expression. The Clock/Bmal1 heterodimer recruits the KATs, p300/CBP, to facilitate transactivation at target genes (Takahata *et al.*, 2000). In addition, association of KDACs and their complexes with clock-regulated gene promoters has been shown to exhibit a rhythmic periodicity (Duong *et al.*, 2011; Feng *et al.*, 2011; Kim *et al.*, 2014). Accordingly, rhythmic histone acetylation has been observed at promoters of several core clock components, including *Per1*, *Per2*, and *Cry1* (Etchegaray *et al.*, 2003). In the case of the *Per1* gene, increased histone acetylation at its promoter has been shown to activate transcription (Naruse *et al.*, 2004). Clock itself has intrinsic KAT activity that targets its binding partner, Bmal1, in a

rhythmic fashion. Bmal1 acetylation leads to the recruitment of Cry1 and subsequent gene repression (Hirayama *et al.*, 2007; Doi *et al.*, 2006). Sirt1, a Class III, NAD⁺-dependent KDAC, is required for high-magnitude circadian expression of several core clock genes and has been shown to balance the KAT activity of Clock (Nakahata *et al.*, 2008; Asher *et al.*, 2008). Zinc-dependent Class I KDACs also contribute to circadian clock physiology. The KDAC1/2-containing Sin3 and Nucleosome Remodeling and Deacetylase (NuRD) complexes associate with promoters of *Per* and *Cry* genes in rhythmic fashion to repress their transcription (Kim *et al.*, 2014; Naruse *et al.*, 2004; Duong *et al.*, 2014). Finally, Rev-erba (encoded by the *Nr1d1* gene) has been found to recruit the NCoR/KDAC3 complex to repress *Bmal1* transcription (Yin *et al.*, 2005). In mouse liver, oscillatory association of Rev-erba and KDAC3 with genes that regulate lipid metabolism contributes to hepatic lipid homeostasis (Feng *et al.*, 2011). Thus, reversible acetylation at histones and non-histone proteins controlled by KATs and KDACs is an integral part of the circadian transcription-translation feedback loop.

Valproic acid (VPA) is a carboxylate derivative of valeric acid prescribed over 7 million times per year as of 2014 (<http://clincalc.com/DrugStats/Top200Drugs.aspx>; <https://www.ahrq.gov/research/data/meps/index.html>). It causes both acute and chronic, reversible hepatotoxicity [reviewed in (Cotariu *et al.*, 1988; Nanau *et al.*, 2013; Chang *et al.*, 2006)]. After two decades of clinical use, VPA was reported to be a KDAC inhibitor (KDACi) (Gottlicher *et al.*, 2001). VPA has been shown to cause metabolic side effects in about 50% of regular users, including non-alcoholic fatty liver disease, hyperinsulinemia, hyperlipidemia, and weight gain that leads to increased body mass index, among others (Luef *et al.*, 2009; Perucca, 2002). Disruption of circadian rhythm is also linked to several metabolic abnormalities, including cardiovascular disease and metabolic syndrome with characteristics of hyperlipidemia, hepatic steatosis, reduced insulin sensitivity, and obesity [reviewed in (Eckel-Mahan *et al.*, 2013; Froy, 2011)]. The similarities in side effects between chronic VPA usage and circadian rhythm disruption and the emerging roles of KATs and KDACs in regulating circadian transcription feedback loops, raise the possibility that VPA may disrupt clock function. A previous study showed that oscillatory activity of an integrated *PER2* promoter driving expression of the luciferase gene (*PER2::LUC*) was phase-shifted by VPA treatment in tissue explants of mouse SCN and in cultured human fibroblasts (Johansson *et al.*, 2011). In the current study we investigated whether VPA exposure impacts the expression of genes encoding multiple core circadian transcription factors (TFs) in a cell culture model system. In cells that were not synchronized to the circadian clock, we showed that treatment with VPA or another clinically-relevant KDACi, vorinostat, simultaneously increased the expression of genes encoding circadian TFs that regulate the positive and negative limbs of the circadian clock. In cells synchronized to the circadian clock, we found that VPA exposure disrupts the oscillatory expression pattern of genes encoding TFs regulating both limbs of the circadian transcription-translation feedback loop. These results suggest that the common side effects of VPA could be mediated in part through disruptions to circadian rhythm.

MATERIALS AND METHODS

Cell lines, reagents, and antibodies

Murine hepatoma cells (Hepa-1c1c7) and murine fibroblast cells (NIH/3T3) were obtained from American Type Culture Collection and maintained in Minimum Essential Media α (MEM α) and Dulbecco's Modified Eagle Medium (Invitrogen), respectively, containing 10% fetal bovine serum (FBS) (Peak Serum) and 0.1% gentamicin (Invitrogen). Dexamethasone (Dex) and valproic acid (VPA) were obtained from Sigma-Aldrich. Anti-acetylated histone H3 (06–599), H3K27ac (07–360), and acetylated histone H4 (06–866) were all obtained from Millipore. Anti-histone H3 (Finlay *et al.*, 1991) was obtained from Cell Signaling. The secondary goat anti-rabbit antibody (111–035-144) was purchased from Jackson ImmunoResearch.

RNA Analysis

Hepa-1c1c7 or NIH/3T3 cells were seeded in 6 well dishes at 2.5×10^5 cells per well. After 24 h, cells were treated with VPA (5mM) for 5 h or 24 h followed by lysis in Trizol (Invitrogen). Total RNA was isolated according to the manufacturer's protocol using the Nucleospin RNA II kit (Clontech) and included DNase I digestion. cDNA was generated using the qScript cDNA synthesis kit (Quanta). qPCR was performed using a StepOne Plus instrument (Applied Biosystems) with SYBR Green mastermix (Quanta) according to the manufacturer's specifications. Table 1 lists the gene-specific exon-exon and exon-intron primer pairs used to measure levels of mRNA and nascent transcripts, respectively. In each experiment the test gene Ct values were normalized against the geometric mean of the corresponding glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) and hypoxanthine guanine phosphoribosyltransferase (*Hprt*) Ct values to obtain Ct values for each sample. These housekeeping genes were chosen for normalization based on our long-term observations that their expression is not affected in these cell lines by treatment with Dex and/or various KDAC inhibitors. To determine changes in expression between treated and untreated samples, the Ct values for treated samples were normalized against those from the untreated controls for each test gene to derive Ct values. In each experiment, average primer efficiency was calculated from standard curves and used to convert the Ct values into fold change. The fold change values were then used to graph results and calculate SEM. The Ct values of untreated samples were compared to treated samples using the paired t test (Graphpad Prism) to determine whether the changes were statistically significant (p 0.05).

Western Blot analysis

Whole cell lysates were prepared by adding 2X SDS-PAGE buffer to cells. After denaturation proteins were separated by SDS-PAGE and transferred onto a nitrocellulose membrane (Bio-Rad) at 400mA for 4 hours. Membranes were blocked with 2% non-fat dry milk in TBS for 1 hour, followed by exposure to primary antibodies at 4°C overnight. After subsequent exposure to secondary antibodies, membranes were washed 3 times with TBS/0.1% Tween-20 solution. The proteins were visualized with enhanced chemiluminescence (ECL) solution using a chemiluminescence imaging system (BioRad).

Per1 sensitivity studies

NIH/3T3 cells were seeded in 6 well dishes at 2.5×10^5 cells per well. After 24 h, cells were treated with Dex (0.1nM, 0.3nM, 1nM, 3nM, 10nM, 30nM, 100nM, 300nM, 1 μ M) for 1 h. Cells were harvested and RNA was isolated and analyzed as described above. The fold change values were fit with a Dose Response curve using Graphpad Prism. Again, a paired t-test was run to determine whether there were significant differences between untreated and Dex treated samples.

Circadian rhythm synchronization

NIH/3T3 cells were seeded in 60 $\times$ 15 mm dishes at 3×10^5 cells per dish. After 3 days of incubation, media was changed to media supplemented with media containing charcoal stripped FBS (1% FBS). After 24 h, cells were treated with Dex (1nM) for 1 h. After treatment, the media was removed and the cells were washed with 1% FBS supplemented media before being replaced with fresh media (1% FBS), which marks the 0 h time point (circadian time 0 or CT0). The cells were then harvested at various time points over the next 48 h and RNA was isolated and analyzed as described above. In experiments that included VPA treatment, VPA (5 mM) was added at the same time as Dex treatment or at Circadian Time 12 (CT12). In the former case, when VPA was added at the same time as Dex, it was re-added with fresh media (1% FBS) after cells were washed to remove Dex.

After RT-qPCR (described above) fold change values were derived based on comparison of treated conditions with the untreated condition (at the time of Dex treatment). The fold change values were fit with the following equation using Graphpad Prism:

$$y = \text{Baseline} + \text{Amplitude} \sin(\text{frequency} \times x + \text{PhaseShift})$$

Using this equation the following parameters were extrapolated from each gene: 1) baseline, a measurement of the average expression level around which the curve oscillate over time and 2) amplitude, the change in expression at high and low points relative to the corresponding baseline. The Ct values of two different treatments (e.g. untreated vs. Dex alone or Dex treatment vs. Dex and VPA cotreatment) were compared using the paired t test to determine whether the changes were statistically significant ($p < 0.05$).

RESULTS

Valproic Acid and vorinostat induce expression of genes encoding circadian rhythm TFs

During the analysis of expression profiling data in multiple cell lines, we noted that KDAC inhibitors (KDACi), including VPA, induced mRNA levels of several transcription factors (TFs) that regulate the circadian clock (not shown). One of the cell lines we examined, Hepa-1c1c7, was derived from mouse hepatoma. Since VPA is established to impact liver function (Cotariu *et al.*, 1988; Nanau *et al.*, 2013; Chang *et al.*, 2006), we used this cell line to verify the impact of VPA on mRNA levels of several TFs vital to the negative (*Per1*, *Per2*, *Cry1*, *Cry2*, *Nr1d1* [Reverba]) and positive (*Bmal1*) limbs of the circadian transcription-translation feedback loop. Fig 1A shows that all circadian genes tested are significantly upregulated by 5 h VPA treatment when compared to untreated cells. To determine whether

this represents a transient response to the drug, we measured expression of these genes after 24 h of VPA treatment. With the exception of *Nr1d1*, all of the genes tested demonstrate sustained upregulation in the presence of VPA.

To determine whether this was a cell-specific response to VPA treatment, we repeated the experiment in NIH/3T3 cells, a standard in the field for examining the circadian rhythm transcription-translation feedback loop *in vitro* (Farnell *et al.*, 2011; Nagoshi *et al.*, 2005). In these cells, neither short- nor long-term VPA exposure affected expression of *Per1* and *Per2* and expression of *Nr1d1* was mildly but significantly decreased. However, as was the case in the Hepa1-c1c7 cells, expression of *Cry1*, *Cry2*, and *Bmal1* were significantly upregulated by 5 h VPA treatment (Fig. 1B). At 24 h VPA treatment, upregulated expression of *Cry1* and *Cry2* was sustained. While VPA impacted expression of more circadian TF genes in Hepa-1c1c7 cells, it caused significant and simultaneous upregulation of genes encoding TFs in the negative (*Cry1* and *Cry2*) and positive (*Bmal1*) limbs of the circadian transcriptional feedback loop.

Since VPA is known to have targets other than Class I KDACs (Terbach *et al.*, 2009), we also exposed both cell lines to vorinostat, another clinically-relevant KDACi. Vorinostat, a hydroxamate, and VPA, a simple aliphatic acid, are structurally distinct and likely to have different off-target effects. At 5 h treatment in Hepa-1c1c7 cells (Fig. 1C), vorinostat caused significant upregulation of 4 of 6 circadian TF genes tested, including three members of the negative limb (*Per2*, *Cry1*, *Cry2*) and *Bmal1*, representing the positive limb. Thus, there is strong overlap in the circadian TF genes upregulated by VPA and vorinostat at 5 h, which captures mostly primary changes in gene expression due to drug treatment. At 24 h of vorinostat treatment, 3 of the 4 genes upregulated at 5 h are still significantly higher than baseline although the magnitude of the difference is lower. In NIH/3T3 cells, vorinostat causes increased expression of the same three genes upregulated by VPA at 5 h (Fig. 1D). *Cry1* and *Bmal1* were significantly up-regulated; *Cry2* expression was upregulated relative to control in each replicate but the changes fell just short of significance by the paired t-test ($p=0.07$). By 24 h of vorinostat treatment, only *Bmal1* upregulation is sustained. In general, at the 5 h treatment time, the effects of VPA and vorinostat are very similar in each cell line, suggesting that early upregulation of the genes encoding the circadian TFs is mediated through the shared target of VPA and vorinostat, Class I KDACs (Khan *et al.*, 2008). Effects of the two KDACi are more divergent at the 24 h time point, which will capture secondary effects of drug treatment.

Finally, to ensure that both VPA and vorinostat display KDAC-inhibiting activity at the concentrations used, we treated Hepa-1c1c7 (Fig. 1E) and NIH/3T3 (Fig. 1F) cells with each drug and assayed acetylation of histones H3 and H4 by Western blot with antibodies against histone H3 acetylated at K27 or at K9/K14 and a pan-acetylH4 antibody. The results show that VPA and vorinostat induce robust increases in both H3 and H4 acetylation at 5 and 24 h treatment.

Low dose glucocorticoid activated circadian rhythm expression of core peripheral clock factors

Because *Bmal1* gene expression is normally antiphase to *Per* and *Cry* gene expression during the circadian cycle (Yagita *et al.*, 2001) the above findings raise the possibility that VPA or vorinostat may disrupt the oscillatory expression of essential circadian genes important for proper clock function. Immortalized, non-transformed cell lines such as NIH/3T3 have been most commonly used to study clock function in vitro (Nagoshi *et al.*, 2005; Balsalobre *et al.*, 2000; Balsalobre *et al.*, 1998). Reddy et al reported that low dose dexamethasone treatment synchronizes the circadian clock because the *Per1* gene is hypersensitive to activation by glucocorticoids and triggers the oscillatory expression of other circadian TFs (Reddy *et al.*, 2009). To determine whether this works in NIH/3T3 cells, we characterized the Dex dose-response relationship for *Per1* and several other glucocorticoid-activated genes and determined the EC₅₀ concentration for each gene. As shown in Fig. 2A, the EC₅₀ for *Per1* is the lowest at 1nM. At this concentration *Per1* is activated while other glucocorticoid-sensitive genes are unaffected.

We previously reported that VPA and other KDACi impair glucocorticoid receptor-activated transcription at up to 50% of target genes (Kadiyala *et al.*, 2013). Because *Per1* activation is vital for the oscillation of other circadian genes, it was important to determine whether KDACi treatment disrupts this activation. NIH/3T3 cells were treated with 1nM Dex for 1 hour in order to activate the *Per1* gene in the presence and absence of VPA, as shown in Fig. 2B. VPA treatment had no effect on the nascent transcript levels of *Per1*.

To determine whether low dose glucocorticoid and *Per1* activation can initiate circadian rhythm, contact-inhibited NIH/3T3 cells were treated with 1nM Dex for 1 hour, after which the Dex-treated media was removed and replaced with low serum media (1% FBS) without Dex. The mRNAs encoding several core peripheral clock genes were monitored over 48 h along with two housekeeping genes (*Gapdh* and *Hprt*). Nonlinear regression was used to curve fit the data to a sine wave with circadian time 0 (CT0) set at the time of Dex removal. As expected, expression of the housekeeping genes does not oscillate (Fig. 3F). In contrast, exposure to low dose Dex induces the oscillatory expression of genes encoding the core circadian TFs (Fig 3A–E). The *Bmal1* gene, belonging to the positive limb, has an oscillatory pattern opposite to those of the *Per2*, *Cry1*, *Cry2*, and *Nr1d1* genes, all of which belong to the negative limb. Within the first 8 h after Dex treatment expression of the latter genes increases, while *Bmal1* mRNA levels significantly decrease. *Bmal1* gene expression peaks between 8 and 16 h and between 32 and 40 h, times when the expression of genes encoding TFs of the negative limb are declining.

Table 2 shows 1) baseline, a measurement of the average expression level around which the curve oscillates over time and 2) amplitude, the change in expression at high points (peaks) and low points (troughs) relative to the corresponding baseline. These values for each of the five circadian TF genes tested in cells synchronized with Dex alone are listed in column 2. The times at which peaks and troughs occurred for each gene over the 48 h period were also determined and are shown for Dex-synchronized cells in the unshaded columns of Tables 3 and 4.

Valproic Acid affects circadian gene expression in a gene-selective fashion

To ascertain whether VPA alters circadian gene oscillation in vitro, we exposed Dex-synchronized NIH/3T3 cells to VPA. VPA was added to the NIH/3T3 cultures at the same time as Dex and was maintained in the medium after Dex washout. To determine whether VPA acts as a KDACi under these conditions, lysates were generated from NIH/3T3 cells just prior to Dex and VPA addition (UT) and at CT1, 8, 24, and 48. Fig. 4F shows that VPA induces a robust increase in histone H3 and H4 acetylation that is maintained through the 48 h treatment period.

To determine whether VPA exposure impacts clock function, levels of mRNA from circadian TF genes were monitored and analyzed as described above. In Fig. 4A–E, the curves generated by Dex treatment alone (Fig. 3A–E) are indicated by a dotted line in each graph for the purpose of comparison. Baselines and amplitudes were extrapolated from the curves for each gene and are listed in Table 2 (shaded columns). Changes in these parameters caused by VPA exposure provide a measure of whether it alters the range of expression for a given gene. The results show that the oscillatory expression of some, but not all, of the circadian TF genes were affected by VPA. Curve-fitting of *Per2* mRNA expression (Fig. 4A) does not indicate any significant impact of VPA treatment on baseline or amplitude [Table 2, compare values in column 3 (VPA,Dex) to those in column 2 (Dex alone)]. For *Nr1d1* mRNA (Fig. 4B), the amplitude for the VPA-treated cells was similar to that observed in cells treated with Dex alone, but baseline expression was mildly but significantly decreased.

Expression of the *Cry1* (Fig 4C) and *Cry2* (Fig 4D) genes were similarly affected by VPA exposure at CT0. While the amplitudes of their oscillatory expression cycles were not changed, their baseline expression was increased by 40% (*Cry1*) and 65% (*Cry2*) (Table 2, compare baselines for Dex alone and VPA, Dex). Thus VPA resets the range of their expression to a higher level. In support of this, fold changes in *Cry1* and *Cry2* mRNAs calculated in the presence of both VPA and Dex (relative to levels of mRNA at the time of initial treatment) were significantly higher than those observed with Dex treatment over much of the 48 h cycle (denoted with pound signs in Fig. 4C and 4D). The cycle of *Bmal1* mRNA expression (Fig. 4E) was altered the most by VPA treatment. While the amplitude was unaffected by VPA exposure at CT0 (Table 2), the baseline expression was significantly reduced by greater than 40%. Thus, in contrast to its effects on *Cry1* and *Cry2* expression, VPA resets the range of *Bmal1* expression to lower level. In contrast to the negative limb circadian TF genes, VPA induced a 6 h phase shift in the oscillatory cycle of *Bmal1* mRNA levels. In the presence of Dex alone, *Bmal1* mRNA levels reached a low point (trough) within 2 h after Dex removal (Table 3, column 4). However, the presence of VPA caused a prolonged decrease in *Bmal1* mRNA that results in the trough being reached at about 8 h after Dex removal (Table 3, column 5). This 6 h phase delay is maintained over a second 24 h cycle as evidenced by a 6 h shift in the times of peaks and troughs for *Bmal1* mRNA when comparing VPA, Dex to Dex alone (Table 3, shaded vs. unshaded columns). These results show that VPA treatment at CT0 has a dramatic effect on the positive limb of the circadian cycle.

Addition of Valproic Acid at CT12 causes phase shifts in expression of genes encoding TFs belonging to both limbs of the circadian transcription-translation feedback loop.

In the experiments described above, cells were exposed to VPA at a time when expression of the TF genes in the negative limb were increasing and *Bmal1* gene expression was decreasing. It is possible that adding VPA 12 h later, when *Bmal1* gene expression is on the rise, may have less of an effect on oscillatory expression cycles. To address this, we treated Dex-synchronized cells with VPA 12 h after Dex was washed out (Circadian Time 12, CT12) and monitored expression of the circadian TF genes. As in Fig. 4, the graphs of Fig. 5 show the curves generated by Dex treatment alone (Fig. 3) as a dotted line. Relative to Dex alone, VPA treatment at CT12 had no effect on baseline expression of *Bmal1* mRNA but significantly increased amplitude by greater than 2-fold (Table 2, compare unshaded columns, CT12 VPA, Dex vs. Dex). It also caused an immediate, robust increase in *Bmal1* mRNA levels before they trended downward after CT16 (Fig 5E). This produced a 3 h phase shift [Table 4, compare peak and trough times of VPA, Dex (shaded columns) with Dex alone]. Although the curve-fitting does not reflect it, there may also be a lengthening of the period between peaks of *Bmal1* expression (Fig. 5E). The first peak occurred at CT16 while the subsequent peak occurred around CT44, a difference of 28 h.

The effects of VPA exposure at CT12 on expression of circadian TF genes in the negative limb were more pronounced than the effects of exposure at CT0. As shown in Fig 5A, the cycle of *Per2* mRNA expression, which was unaffected by VPA exposure around CT0, showed significant decreases in baseline (20%) and amplitude (65%) (Table 2) as well as a 5 h phase shift (Table 4). In the case of *Nr1d1*, (Fig. 5B), VPA at CT12 did not affect baseline expression of the oscillatory cycle but significantly increased amplitude by greater than 2-fold (Table 2) and caused a 3–4 h phase shift (Table 4). The oscillatory expression *Cry1* and *Cry2* mRNAs (Figs. 5C–D) was profoundly impacted by VPA treatment at CT12. The cycle of *Cry1* mRNA expression showed a significant increase in baseline expression (Table 2, CT12 VPA, Dex vs Dex). In addition, there was a phase shift of 12 h (Table 4) resulting in an opposite oscillatory cycle relative to Dex treatment alone (Fig. 5C). In fact, its oscillation pattern becomes similar, rather than opposite, to that of *Bmal1* mRNA (compare solid lines in Figs. 5C and 5E). After VPA treatment at CT12, *Cry2* mRNA essentially loses its oscillatory pattern of expression (Fig. 5D) and, at most time points tested, its average level of expression is significantly higher relative to Dex alone, which is reflected in a significantly higher baseline (Table 2).

DISCUSSION

VPA, a well-established anticonvulsant and mood stabilizer, causes several metabolic disturbances similar to those observed by disruption of circadian rhythm. Two previous *in vitro* studies indicated that VPA or an additional KDACi, trichostatin A (TSA), affected the oscillatory expression of the *Per1* (Naruse *et al.*, 2004) and *Per2* (Johansson *et al.*, 2011) genes, which function in the negative limb of the circadian transcription-translation feedback loop. In the current study we show that exposure to the KDACi, VPA or vorinostat, alters expression of genes encoding multiple core circadian TFs in two murine cell lines of different tissue origin. Expression of the *Cry1*, *Cry2*, and *Bmal1* genes were sensitive to

short-term (5 h) treatment with VPA or vorinostat in both cell lines. The effects of these KDACi were more divergent after 24 h exposure. Although we cannot rule out off-target secondary effects, these differences could be due to the fact that VPA is a Class I-selective KDACi while vorinostat is a pan-KDACi which also efficiently inhibits the Class IIb KDAC6 (Bantscheff *et al.*, 2011). In cells synchronized to the circadian transcription-translation feedback loop through glucocorticoid treatment, VPA exposure perturbs oscillatory expression of genes encoding core circadian TFs that participate in both the positive and negative limbs of the circadian clock. VPA exposure impacts baseline, amplitude, and phase of oscillatory expression in a gene-dependent manner. Also, the effects of VPA exposure are different at the beginning of the circadian cycle (CT0) versus mid-cycle (CT12). These findings suggest that VPA has the potential to disrupt circadian rhythms at the organismal level and alter physiological homeostasis.

Glucocorticoid signaling is thought to be a component of the communication between the master clock in the SCN and the peripheral clocks. Serum glucocorticoid levels peak in the morning and reach a low point in the evening (Krieger *et al.*, 1971; Weitzman *et al.*, 1971). We used low dose glucocorticoid to initiate the rhythmic expression of genes encoding five core circadian TFs through upregulation of the *Per1* gene in NIH/3T3 cells. In addition to the genes shown in Figs. 3–5, *Per1* mRNA was also monitored but, after the initial Dex-induced peak in expression, there were no further oscillations in its expression (data not shown), consistent with the findings of Osland *et al.* (Osland *et al.*, 2011). *Clock* mRNA was not examined because it does not oscillate during the circadian cycle in cultured fibroblasts (Osland *et al.*, 2011; Yagita *et al.*, 2001). In mouse liver *Clock* mRNA and protein show shallow oscillations (Ripperger *et al.*, 2006; Preitner *et al.*, 2002). Instead, *Clock* protein undergoes robust changes in post-translational modifications during the circadian cycle (Lee *et al.*, 2001).

Our results in cells not synchronized to the circadian clock suggest that both VPA and vorinostat may disrupt the circadian transcriptional feedback cycle. However, the two drugs have very different pharmacokinetic properties and dosing schedules. In humans vorinostat is rapidly cleared from serum with a half-life of 1.5–2 h (Rubin *et al.*, 2006) while VPA persists with a half-life in serum of 8–20 h (Perucca, 2002; Johannessen *et al.*, 2003). In addition, vorinostat, approved for use against advanced cutaneous T cell lymphoma, is typically given once daily while VPA is taken 1–3X daily. Its extended half-life combined with multiple daily dosing makes VPA a more likely candidate for disruption of the circadian clock because it probably persists in human serum over a 24 h cycle, especially if patients are taking multiple daily doses.

VPA was initially added at the beginning of the circadian cycle to model VPA users who take a daily dose in the mornings when serum glucocorticoid levels are high. After Dex washout, it was maintained in the medium to model users taking multiple daily doses. In this case oscillatory expression of genes encoding TFs in both the positive and negative limbs of the circadian feedback loop were affected. Baseline expression of *Cry1* and *Cry2* genes was significantly increased while that of the *Bmal1* gene was decreased. This could shift the balance of expression between circadian TFs towards those in the negative limb of the circadian cycle. In addition, the cycle of *Bmal1* gene expression was phase delayed by 6 h,

putting its oscillatory expression out of sync with expression of genes encoding TFs that drive the negative limb of the circadian cycle because their phases of expression were unaffected. We also measured VPA exposure at CT12, which would be analogous to a VPA user taking a dose in the evening. This had a stronger effect, causing multiple changes in the rhythmic cycling of TF genes in both negative and positive limbs of the circadian feedback loop. Similar to VPA exposure at CT0, baseline expression of the *Cry1* and *Cry2* genes was significantly increased. In addition, *Cry1* gene expression was phase advanced by 12 h and *Cry2* gene expression lost its rhythmicity. Both *Cry1* and *Cry2* have been implicated in maintaining period length and circadian rhythm, and their delicate balance of expression is crucial for proper circadian function (van der Horst *et al.*, 1999).

While unaffected by VPA exposure at CT0, baseline expression of the *Per2* gene was significantly reduced and its expression was phase-delayed by approximately 5 h when VPA exposure occurred at CT12. This finding agrees with that of Johansson *et al.*, who demonstrated that the magnitude of VPA-induced phase-shifts in circadian *Per2* promoter activity was dependent on the time of VPA exposure (Johansson *et al.*, 2011). In the case of *Bmal1* gene expression, its amplitude was increased by about 50% and its phase was delayed by 3 h. Since the phase of *Nr1d1* gene expression was similarly delayed, the oscillatory expression of these two genes remains opposite. However, due to the large phase advance of *Cry1* gene expression, VPA exposure at CT12 caused expression of the *Bmal1* and *Cry1* genes to peak at almost the same time rather than *Bmal1* being antiphase to *Cry1* in the presence of Dex alone (Table 4). The large *Cry1* phase shift also puts it out of the normal sync with *Per2* gene expression, which peaks about 3 h before *Cry1* in the presence of Dex alone (Table 4).

The differences in response of the circadian TF genes to VPA exposure at CT0 and CT12 are likely a reflection of the transcription complexes present at their promoters at those times. For example, at CT0 in the presence of Dex alone, *Bmal1* expression was trending downward, possibly due to the inhibitory action of Rev-erba proteins at its promoter. VPA exposure causes a prolonged downward trend in *Bmal1* gene expression under these circumstances. However, at CT12 *Bmal1* expression is on an upward cycle, possibly due to ROR proteins at its promoter. VPA exposure at this time causes a large increase in *Bmal1* expression. The exact mechanisms behind these differences are unclear but deserve further study.

While VPA has several mechanisms of action (Terbach *et al.*, 2009), its effects on transcription may be due, at least in part, to its ability to inhibit KDACs and thereby alter the acetylation state of both histones and transcriptional regulatory proteins (Scholz *et al.*, 2015). Our comparison of VPA and vorinostat showed that both induced histone acetylation (Figs. 1E, 1F) in unsynchronized Hepa-1c1c7 and NIH/3T3 cells. In addition, expression of TF genes in both the positive and negative limbs of the circadian feedback loop was induced by VPA or vorinostat (Fig. 1A–D). We also showed that VPA treatment induces robust increases in histone acetylation over the 48 h treatment period in clock-synchronized NIH/3T3 cells. The study of Johansson *et al.* found that VPA and a second KDACi, TSA, had very similar effects on rhythmic PER2::LUC gene expression in SCN tissue explants and cultured primary fibroblasts (Johansson *et al.*, 2011). The hydroxamic acids, vorinostat

and TSA, have very different structures compared to VPA, a simple aliphatic acid, and are likely to have distinct off-target effects. Thus, their similar effects on circadian TF gene expression in this study and on PER2::LUC expression in the Johansson study (Johansson *et al.*, 2011) suggest that they are mediated through their shared targets, the zinc-dependent KDACs. In further support of this, Johansson *et al.* tested the effects of inhibitors of various pathways that VPA has been shown to modulate (e.g. MAPK, JNK, GABA signaling) (Terbach *et al.*, 2009) and found that they did not block any of the effects of VPA on the amplitude and phase of PER2::LUC expression in cultured human fibroblasts (Johansson *et al.*, 2011).

Altered amplitudes and phase shifts of circadian TF expression have been documented in various disease states and in aging. Decreased rhythm amplitudes have been found in patients suffering from depression as well as from bipolar disorder (Yang *et al.*, 2009; Souetre *et al.*, 1989). Older rodents have also been shown to have reduced circadian rhythm amplitudes compared to younger rodents (Kolker *et al.*, 2003). In fact, systems with robust circadian amplitudes are more resistant to phase perturbation than those with lower amplitudes (Pittendrigh *et al.*, 1991). Therefore, the reduction of circadian amplitude may cause instability of circadian rhythm and other homeostatic processes (Tao *et al.*, 2015; Monk, 2005). Familial advanced sleep phase syndrome is a circadian disruption disorder where patients experience early sleep onset and delayed awakening (Toh *et al.*, 2001). Phase advances are also implicated in the process of aging, as well as decreased tolerance to abrupt phase shift changes (Monk, 2005). Due to modern-day lifestyles, many people experience possible phase shifts on a daily basis. In fact, certain stimuli, including light, cause phase shifts through increased Per levels that subsequently reset the phases of other genes in the feedback loop (Kolker *et al.*, 2003). Based on our study and that of Johansson, VPA is a phase-setting agent, causing shifts when added at various points of the circadian cycle (Johansson *et al.*, 2011). Our study clearly shows that VPA exposure mid-cycle, at CT12, caused major phase shifts for all core clock components. However, even when added at CT0, VPA causes a major phase delay in oscillatory *Bmal1* gene expression and a significant decline in baseline expression. Deletion of a single clock gene rarely causes complete loss of rhythmicity (Zheng *et al.*, 2001; van der Horst *et al.*, 1999) with the exception of *Bmal1*; knockout of *Bmal1* in mice severely disrupts circadian rhythm with a complete loss of rhythmicity in constant darkness as well as locomotor impairment (Bunger *et al.*, 2000). Thus, disruptions in circadian *Bmal1* expression alone may lead to robust downstream physiological effects.

VPA has been in clinical use for almost 40 years for a number of disease states including seizure, mood disorders, and migraines. With a half-life in humans of 8–20 hours, patients taking multiple daily doses likely maintain some serum level of VPA throughout a 24 h period (Perucca, 2002). Our study shows that VPA exposure has profound effects on negative and positive limbs of the circadian transcriptional feedback loop *in vitro*. The approval of 4 KDACi for clinical use against cancer in the last decade has spurred evaluation these drugs for use in other diseases (Haberland *et al.*, 2009); thus, human exposure to these drugs is likely to increase. In light of this, the circadian impact of clinically relevant KDAC inhibitors, such as VPA, is worthy of further study. A recent study cataloged genes that showed oscillatory expression in a variety of tissues in mice (Zhang *et al.*, 2014). Among

these genes were those encoding the targets of over 50% of all drugs approved for clinical use. To reduce toxicities associated with xenobiotic exposure, it is essential to understand how therapeutics such as VPA impact circadian rhythm and to determine when, during the 24 h cycle, they could be given with minimal negative circadian effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

We are grateful to Ms. Molly Simon (University of Arizona) for assistance with curve-fitting and to Dean Billheimer, PhD (University of Arizona) for statistical help. We also thank Drs. Walt Klimecki and Aikseng Ooi (University of Arizona) for critical reading of the manuscript.

Funding for the study came from the following sources: National Science Foundation (MCB-1122038) to CLS, ALSAM Foundation Skaggs Scholars grant program to CLS, NIEHS T32 ES007091 (PI – Nathan Cherrington, PhD), and the Southwest Environmental Health Sciences Center (P30 ES006694, PI-Nathan Cherrington, PhD).

References

- Albrecht U, 2012 Timing to perfection: the biology of central and peripheral circadian clocks. *Neuron* 74, 246–260. [PubMed: 22542179]
- Asher G, Gatfield D, Stratmann M, Reinke H, Dibner C, Kreppel F, Mostoslavsky R, Alt FW, and Schibler U, 2008 SIRT1 regulates circadian clock gene expression through PER2 deacetylation. *Cell* 134, 317–328. [PubMed: 18662546]
- Balsalobre A, Brown SA, Marcacci L, Tronche F, Kellendonk C, Reichardt HM, Schutz G, and Schibler U, 2000 Resetting of circadian time in peripheral tissues by glucocorticoid signaling. *Science* 289, 2344–2347. [PubMed: 11009419]
- Balsalobre A, Damiola F, and Schibler U, 1998 A serum shock induces circadian gene expression in mammalian tissue culture cells. *Cell* 93, 929–937. [PubMed: 9635423]
- Bantscheff M, Hopf C, Savitski MM, Dittmann A, Grandi P, Michon AM, Schlegl J, Abraham Y, Becher I, Bergamini G, Boesche M, Delling M, Dumpelfeld B, Eberhard D, Huthmacher C, Mathieson T, PoECKel D, Reader V, Strunk K, Sweetman G, Kruse U, Neubauer G, Ramsden NG, and Drewes G, 2011 Chemoproteomics profiling of HDAC inhibitors reveals selective targeting of HDAC complexes. *Nat.Biotechnol* 29, 255–265. [PubMed: 21258344]
- Bunger MK, Wilsbacher LD, Moran SM, Clendenin C, Radcliffe LA, Hogenesch JB, Simon MC, Takahashi JS, and Bradfield CA, 2000 Mop3 is an essential component of the master circadian pacemaker in mammals. *Cell* 103, 1009–1017. [PubMed: 11163178]
- Castanon-Cervantes O, Wu M, Ehlen JC, Paul K, Gamble KL, Johnson RL, Besing RC, Menaker M, Gewirtz AT, and Davidson AJ, 2010 Dysregulation of inflammatory responses by chronic circadian disruption. *J.Immunol* 185, 5796–5805. [PubMed: 20944004]
- Chang TK, and Abbott FS, 2006 Oxidative stress as a mechanism of valproic acid-associated hepatotoxicity. *Drug Metab. Rev* 38, 627–639. [PubMed: 17145692]
- Cotariu D, and Zaidman JL, 1988 Valproic acid and the liver. *Clin.Chem* 34, 890–897. [PubMed: 3131043]
- Doi M, Hirayama J, and Sassone-Corsi P, 2006 Circadian regulator CLOCK is a histone acetyltransferase. *Cell* 125, 497–508. [PubMed: 16678094]
- Duong HA, Robles MS, Knutti D, and Weitz CJ, 2011 A molecular mechanism for circadian clock negative feedback. *Science* 332, 1436–1439. [PubMed: 21680841]
- Duong HA, and Weitz CJ, 2014 Temporal orchestration of repressive chromatin modifiers by circadian clock Period complexes. *Nat.Struct.Mol.Biol* 21, 126–132. [PubMed: 24413057]
- Eckel-Mahan K, and Sassone-Corsi P, 2013 Metabolism and the circadian clock converge. *Physiol. Rev* 93, 107–135. [PubMed: 23303907]

- Etchegaray JP, Lee C, Wade PA, and Reppert SM, 2003 Rhythmic histone acetylation underlies transcription in the mammalian circadian clock. *Nature* 421, 177–182. [PubMed: 12483227]
- Farnell YF, Shende VR, Neuendorff N, Allen GC, and Earnest DJ, 2011 Immortalized cell lines for real-time analysis of circadian pacemaker and peripheral oscillator properties. *Eur.J.Neurosci* 33, 1533–1540. [PubMed: 21366728]
- Feng D, Liu T, Sun Z, Bugge A, Mullican SE, Alenghat T, Liu XS, and Lazar MA, 2011 A circadian rhythm orchestrated by histone deacetylase 3 controls hepatic lipid metabolism. *Science* 331, 1315–1319. [PubMed: 21393543]
- Finlay DR, Meier E, Bradley P, Horecka J, and Forbes DJ, 1991 A complex of nuclear pore proteins required for pore function. *J.Cell Biol* 114, 169–183. [PubMed: 2050741]
- Froy O, 2011 The circadian clock and metabolism. *Clin.Sci.(Lond)* 120, 65–72. [PubMed: 20929440]
- Garrett RW, and Gasiewicz TA, 2006 The aryl hydrocarbon receptor agonist 2,3,7,8-tetrachlorodibenzo-p-dioxin alters the circadian rhythms, quiescence, and expression of clock genes in murine hematopoietic stem and progenitor cells. *Mol.Pharmacol* 69, 2076–2083. [PubMed: 16556773]
- Gottlicher M, Minucci S, Zhu P, Kramer OH, Schimpf A, Giavara S, Sleeman JP, Lo CF, Nervi C, Pelicci PG, and Heinzel T, 2001 Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. *EMBO J* 20, 6969–6978. [PubMed: 11742974]
- Haberland M, Montgomery RL, and Olson EN, 2009 The many roles of histone deacetylases in development and physiology: implications for disease and therapy. *Nat.Rev.Genet* 10, 32–42. [PubMed: 19065135]
- Hirayama J, Sahar S, Grimaldi B, Tamaru T, Takamatsu K, Nakahata Y, and Sassone-Corsi P, 2007 CLOCK-mediated acetylation of BMAL1 controls circadian function. *Nature* 450, 1086–1090. [PubMed: 18075593]
- Iurisci I, Filipski E, Sallam H, Harper F, Guettier C, Maire I, Hassan M, Iacobelli S, and Levi F, 2009 Liver circadian clock, a pharmacologic target of cyclin-dependent kinase inhibitor seliciclib. *Chronobiol.Int* 26, 1169–1188. [PubMed: 19731111]
- Jimenez-Ortega V, Cano BP, Fernandez-Mateos P, Cardinali DP, and Esquifino AI, 2012 Cadmium as an endocrine disruptor: correlation with anterior pituitary redox and circadian clock mechanisms and prevention by melatonin. *Free Radic.Biol.Med* 53, 2287–2297. [PubMed: 23085516]
- Jin X, Shearman LP, Weaver DR, Zylka MJ, de Vries GJ, and Reppert SM, 1999 A molecular mechanism regulating rhythmic output from the suprachiasmatic circadian clock. *Cell* 96, 57–68. [PubMed: 9989497]
- Johannessen CU, and Johannessen SI, 2003 Valproate: past, present, and future. *CNS Drug Rev.* 9, 199–216. [PubMed: 12847559]
- Johansson AS, Brask J, Owe-Larsson B, Hetta J, and Lundkvist GB, 2011 Valproic acid phase shifts the rhythmic expression of Period2::Luciferase. *J.Biol.Rhythms* 26, 541–551. [PubMed: 22215612]
- Kadiyala V, Patrick NM, Mathieu W, Jaime-Frias R, Pookhao N, An L, and Smith CL, 2013 Class I Lysine Deacetylases Facilitate Glucocorticoid-induced Transcription. *J.Biol.Chem* 288, 28900–28912. [PubMed: 23946490]
- Karlsson B, Knutsson A, and Lindahl B, 2001 Is there an association between shift work and having a metabolic syndrome? Results from a population based study of 27,485 people. *Occup.Environ.Med* 58, 747–752. [PubMed: 11600731]
- Khan N, Jeffers M, Kumar S, Hackett C, Boldog F, Khramtsov N, Qian X, Mills E, Berghs SC, Carey N, Finn PW, Collins LS, Tumber A, Ritchie JW, Jensen PB, Lichenstein HS, and Sehested M, 2008 Determination of the class and isoform selectivity of small-molecule histone deacetylase inhibitors. *Biochem.J* 409, 581–589. [PubMed: 17868033]
- Kim JY, Kwak PB, and Weitz CJ, 2014 Specificity in circadian clock feedback from targeted reconstitution of the NuRD corepressor. *Mol.Cell* 56, 738–748. [PubMed: 25453762]
- Kolker DE, Fukuyama H, Huang DS, Takahashi JS, Horton TH, and Turek FW, 2003 Aging alters circadian and light-induced expression of clock genes in golden hamsters. *J.Biol.Rhythms* 18, 159–169. [PubMed: 12693870]

- Koyanagi S, and Ohdo S, 2002 Alteration of intrinsic biological rhythms during interferon treatment and its possible mechanism. *Mol.Pharmacol* 62, 1393–1399. [PubMed: 12435807]
- Krieger DT, Allen W, Rizzo F, and Krieger HP, 1971 Characterization of the normal temporal pattern of plasma corticosteroid levels. *J.Clin.Endocrinol.Metab* 32, 266–284. [PubMed: 4321505]
- Kume K, Zylka MJ, Sriram S, Shearman LP, Weaver DR, Jin X, Maywood ES, Hastings MH, and Reppert SM, 1999 mCRY1 and mCRY2 are essential components of the negative limb of the circadian clock feedback loop. *Cell* 98, 193–205. [PubMed: 10428031]
- Lee C, Etchegaray JP, Cagampang FR, Loudon AS, and Reppert SM, 2001 Posttranslational mechanisms regulate the mammalian circadian clock. *Cell* 107, 855–867. [PubMed: 11779462]
- Li XM, and Levi F, 2007 Circadian physiology is a toxicity target of the anticancer drug gemcitabine in mice. *J.Biol.Rhythms* 22, 159–166. [PubMed: 17440217]
- Li XM, Vincenti M, and Levi F, 2002 Pharmacological effects of vinorelbine on body temperature and locomotor activity circadian rhythms in mice. *Chronobiol.Int* 19, 43–55. [PubMed: 11962685]
- Luef G, Rauchenzauner M, Waldmann M, Sturm W, Sandhofer A, Seppi K, Trinka E, Unterberger I, Ebenbichler CF, Joannidis M, Walser G, Bauer G, Hoppichler F, and Lechleitner M, 2009 Non-alcoholic fatty liver disease (NAFLD), insulin resistance and lipid profile in antiepileptic drug treatment. *Epilepsy Res.* 86, 42–47. [PubMed: 19464851]
- McHill AW, and Wright KP Jr., 2017 Role of sleep and circadian disruption on energy expenditure and in metabolic predisposition to human obesity and metabolic disease. *Obes.Rev* 18 Suppl. 1, 15–24. [PubMed: 28164449]
- Monk TH, 2005 Aging human circadian rhythms: conventional wisdom may not always be right. *J.Biol.Rhythms* 20, 366–374. [PubMed: 16077155]
- Morikawa Y, Nakagawa H, Miura K, Soyama Y, Ishizaki M, Kido T, Naruse Y, Suwazono Y, and Nogawa K, 2005 Shift work and the risk of diabetes mellitus among Japanese male factory workers. *Scand.J.Work Environ.Health* 31, 179–183. [PubMed: 15999569]
- Nagoshi E, Brown SA, Dibner C, Kornmann B, and Schibler U, 2005 Circadian gene expression in cultured cells. *Methods Enzymol.* 393, 543–557. [PubMed: 15817311]
- Nakahata Y, Kaluzova M, Grimaldi B, Sahar S, Hirayama J, Chen D, Guarente LP, and Sassone-Corsi P, 2008 The NAD⁺-dependent deacetylase SIRT1 modulates CLOCK-mediated chromatin remodeling and circadian control. *Cell* 134, 329–340. [PubMed: 18662547]
- Nanau RM, and Neuman MG, 2013 Adverse drug reactions induced by valproic acid. *Clin.Biochem* 46, 1323–1338. [PubMed: 23792104]
- Naruse Y, Oh-hashii K, Iijima N, Naruse M, Yoshioka H, and Tanaka M, 2004 Circadian and light-induced transcription of clock gene *Per1* depends on histone acetylation and deacetylation. *Mol.Cell Biol* 24, 6278–6287. [PubMed: 15226430]
- Ortiz-Tudela E, Iurisci I, Beau J, Karaboue A, Moreau T, Rol MA, Madrid JA, Levi F, and Innominato PF, 2014 The circadian rest-activity rhythm, a potential safety pharmacology endpoint of cancer chemotherapy. *Int.J.Cancer* 134, 2717–2725. [PubMed: 24510611]
- Osland TM, Ferno J, Havik B, Heuch I, Ruoff P, Laerum OD, and Steen VM, 2011 Lithium differentially affects clock gene expression in serum-shocked NIH-3T3 cells. *J.Psychopharmacol* 25, 924–933. [PubMed: 20837565]
- Partch CL, Green CB, and Takahashi JS, 2014 Molecular architecture of the mammalian circadian clock. *Trends Cell Biol.* 24, 90–99. [PubMed: 23916625]
- Perucca E, 2002 Pharmacological and therapeutic properties of valproate: a summary after 35 years of clinical experience. *CNS Drugs* 16, 695–714. [PubMed: 12269862]
- Pittendrigh CS, Kyner WT, and Takamura T, 1991 The amplitude of circadian oscillations: temperature dependence, latitudinal clines, and the photoperiodic time measurement. *J.Biol.Rhythms* 6, 299–313. [PubMed: 1773097]
- Preitner N, Damiola F, Lopez-Molina L, Zakany J, Duboule D, Albrecht U, and Schibler U, 2002 The orphan nuclear receptor REV-ERB α controls circadian transcription within the positive limb of the mammalian circadian oscillator. *Cell* 110, 251–260. [PubMed: 12150932]
- Reddy TE, Pauli F, Sprouse RO, Neff NF, Newberry KM, Garabedian MJ, and Myers RM, 2009 Genomic determination of the glucocorticoid response reveals unexpected mechanisms of gene regulation. *Genome Res.* 19, 2163–2171. [PubMed: 19801529]

- Ripperger JA, and Schibler U, 2006 Rhythmic CLOCK-BMAL1 binding to multiple E-box motifs drives circadian Dbp transcription and chromatin transitions. *Nat.Genet* 38, 369–374. [PubMed: 16474407]
- Rubin EH, Agrawal NG, Friedman EJ, Scott P, Mazina KE, Sun L, Du L, Ricker JL, Frankel SR, Gottesdiener KM, Wagner JA, and Iwamoto M, 2006 A study to determine the effects of food and multiple dosing on the pharmacokinetics of vorinostat given orally to patients with advanced cancer. *Clin.Cancer Res* 12, 7039–7045. [PubMed: 17145826]
- Sato TK, Panda S, Miraglia LJ, Reyes TM, Rudic RD, McNamara P, Naik KA, FitzGerald GA, Kay SA, and Hogenesch JB, 2004 A functional genomics strategy reveals Rora as a component of the mammalian circadian clock. *Neuron* 43, 527–537. [PubMed: 15312651]
- Scholz C, Weinert BT, Wagner SA, Beli P, Miyake Y, Qi J, Jensen LJ, Streicher W, McCarthy AR, Westwood NJ, Lain S, Cox J, Matthias P, Mann M, Bradner JE, and Choudhary C, 2015 Acetylation site specificities of lysine deacetylase inhibitors in human cells. *Nat.Biotechnol* 33, 415–423. [PubMed: 25751058]
- Sen A, and Sellix MT, 2016 The Circadian Timing System and Environmental Circadian Disruption: From Follicles to Fertility. *Endocrinology* 157, 3366–3373. [PubMed: 27501186]
- Shearman LP, Sriram S, Weaver DR, Maywood ES, Chaves I, Zheng B, Kume K, Lee CC, van der Horst GT, Hastings MH, and Reppert SM, 2000 Interacting molecular loops in the mammalian circadian clock. *Science* 288, 1013–1019. [PubMed: 10807566]
- Souetre E, Salvati E, Belugou JL, Pringuey D, Candito M, Krebs B, Ardisson JL, and Darcourt G, 1989 Circadian rhythms in depression and recovery: evidence for blunted amplitude as the main chronobiological abnormality. *Psychiatry Res.* 28, 263–278. [PubMed: 2762432]
- Takahata S, Ozaki T, Mimura J, Kikuchi Y, Sogawa K, and Fujii-Kuriyama Y, 2000 Transactivation mechanisms of mouse clock transcription factors, mClock and mArnt3. *Genes Cells* 5, 739–747. [PubMed: 10971655]
- Tao W, Wu J, Zhang Q, Lai SS, Jiang S, Jiang C, Xu Y, Xue B, Du J, and Li CJ, 2015 EGR1 regulates hepatic clock gene amplitude by activating Per1 transcription. *Sci.Rep* 5, 15212. [PubMed: 26471974]
- Terazono H, Hamdan A, Matsunaga N, Hayasaka N, Kaji H, Egawa T, Makino K, Shigeyoshi Y, Koyanagi S, and Ohdo S, 2008 Modulatory effects of 5-fluorouracil on the rhythmic expression of circadian clock genes: a possible mechanism of chemotherapy-induced circadian rhythm disturbances. *Biochem.Pharmacol* 75, 1616–1622. [PubMed: 18329632]
- Terbach N, and Williams RS, 2009 Structure-function studies for the panacea, valproic acid. *Biochem.Soc.Trans* 37, 1126–1132. [PubMed: 19754465]
- Toh KL, Jones CR, He Y, Eide EJ, Hinz WA, Virshup DM, Ptacek LJ, and Fu YH, 2001 An hPer2 phosphorylation site mutation in familial advanced sleep phase syndrome. *Science* 291, 1040–1043. [PubMed: 11232563]
- van der Horst GT, Muijtjens M, Kobayashi K, Takano R, Kanno S, Takao M, de WJ, Verkerk A, Eker AP, van LD, Buijs R, Bootsma D, Hoeijmakers JH, and Yasui A, 1999 Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms. *Nature* 398, 627–630. [PubMed: 10217146]
- Weitzman ED, Fukushima D, Nogeire C, Roffwarg H, Gallagher TF, and Hellman L, 1971 Twenty-four hour pattern of the episodic secretion of cortisol in normal subjects. *J.Clin.Endocrinol.Metab* 33, 14–22. [PubMed: 4326799]
- Xu CX, Krager SL, Liao DF, and Tischkau SA, 2010 Disruption of CLOCK-BMAL1 transcriptional activity is responsible for aryl hydrocarbon receptor-mediated regulation of Period1 gene. *Toxicol.Sci* 115, 98–108. [PubMed: 20106950]
- Yagita K, Tamanini F, van der Horst GT, and Okamura H, 2001 Molecular mechanisms of the biological clock in cultured fibroblasts. *Science* 292, 278–281. [PubMed: 11303101]
- Yang S, Van Dongen HP, Wang K, Berrettini W, and Bucan M, 2009 Assessment of circadian function in fibroblasts of patients with bipolar disorder. *Mol.Psychiatry* 14, 143–155. [PubMed: 18301395]
- Yin L, and Lazar MA, 2005 The orphan nuclear receptor Rev-erbalph α recruits the N-CoR/histone deacetylase 3 corepressor to regulate the circadian Bmal1 gene. *Mol.Endocrinol* 19, 1452–1459. [PubMed: 15761026]

- Zhang R, Lahens NF, Ballance HI, Hughes ME, and Hogenesch JB, 2014 A circadian gene expression atlas in mammals: implications for biology and medicine. *Proc.Natl.Acad.Sci.U.S.A* 111, 16219–16224. [PubMed: 25349387]
- Zheng B, Albrecht U, Kaasik K, Sage M, Lu W, Vaishnav S, Li Q, Sun ZS, Eichele G, Bradley A, and Lee CC, 2001 Nonredundant roles of the mPer1 and mPer2 genes in the mammalian circadian clock. *Cell* 105, 683–694. [PubMed: 11389837]
- Zheng B, Larkin DW, Albrecht U, Sun ZS, Sage M, Eichele G, Lee CC, and Bradley A, 1999 The mPer2 gene encodes a functional component of the mammalian circadian clock. *Nature* 400, 169–173. [PubMed: 10408444]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Highlights

- VPA induces circadian transcription factor gene expression in cultured cell lines.
- VPA alters rhythmic circadian expression of these transcription factor genes.
- Specific effects of VPA exposure on rhythmic expression depend on circadian time.
- These findings raise the possibility that VPA disrupts circadian rhythms in humans.

Figure 1. KDAC inhibitors induce expression of genes encoding circadian rhythm transcription factors.

(A, C) Hepa-1c1c7 cells were untreated (control) or treated with VPA (5mM) (A) or Vorinostat (2μM) (C) for 5 or 24 h. (B, D) NIH/3T3 were untreated (control), or treated with VPA (B) or Vorinostat (2μM) (D) for 5h or 24h. RNA was isolated and subjected to RT-qPCR with exon-exon primers to measure mRNA. The results are represented as fold change of drug-treated relative to untreated or vehicle-treated. All graphs are a summary of 3–6 independent replicates. Error bars represent standard error of the mean (SEM). * – $p < 0.05$, ** – $p < 0.01$, *** – $p < 0.001$. Asterisks (*) represent significant changes between untreated cells and cells treated with drug. (E, F) Hepa-1c1c7 (E) and NIH/3T3 (F) cells were treated with VPA (5mM) or Vorinostat (2μM) for 0, 5, or 24h. Whole cell lysates were subjected to SDS-PAGE and immunoblotting with antibodies against H3K27Ac, acetylated histone H4, acetylated histone H3, and total histone H3.

Figure 2. *Per1* is hypersensitive to glucocorticoid treatment in the presence or absence of VPA. (A) NIH/3T3 cells were treated for 1 h with increasing concentrations of Dex (0 nM, 0.1nM, 0.3nM, 1nM, 3nM, 10nM, 30nM, 100nM, 300nM or 1 μ M). RNA was isolated and subjected to RT-qPCR with exon-intron primer sets to measure nascent transcripts. Fold changes in nascent transcripts for each treatment concentration relative to levels in untreated cells for *Per1*, *Tgm2*, *Sgk1*, and *Ampd3*. The half maximal effective concentration (EC₅₀) was calculated for each gene tested (shown in the inset). (B). NIH/3T3 cells were treated with Dex (1nM) in the presence or absence of VPA (5 mM) for 1 h. The Dex was then removed by washing and the cells were exposed to serum-free media containing VPA for an additional hour. RNA was isolated and subjected to RT-qPCR. All graphs represent the results of 3–6 independent replicates. Error bars represent standard error of the mean (SEM). * – $p < 0.05$, ** – $p < 0.01$, *** – $p < 0.001$. Asterisks (*) represent significant changes between untreated cells and cells treated with Dex.

Figure 3. Low dose glucocorticoid activates circadian rhythm gene expression of core peripheral clock transcription factors.

NIH/3T3 cells were treated with Dex (1nM) for 1 hr. The cells were then washed and the media was replaced with low-serum (1%) media (referred to as Circadian Time 0, CT0) and gene expression was followed for the next 48 h. RNA was isolated and subjected to RT-qPCR with primer sets to measure the oscillatory pattern of mRNA levels for circadian TF genes (A–E) as well as two housekeeping genes, *Gapdh* and *Hprt* (F). All graphs represent fold changes in expression at various time points relative to expression at the time of Dex addition and are the summary of 7–17 independent biological replicates. Error bars represent standard error of the mean (SEM). Asterisks (*) represent significant changes between expression in untreated cells and in cells treated with Dex. * – $p < 0.05$, ** – $p < 0.01$, *** – $p < 0.001$.

Figure 4. VPA exposure around CT0 affects expression of circadian transcription factor genes in a gene-selective manner.

(A–E) NIH/3T3 cells were treated with Dex (1nM) and VPA (5mM). After 1hr, the cells were washed and the media was replaced with low-serum (1%) media (CT0). For the cells previously treated with VPA, VPA was re-added after Dex removal and maintained for the remainder of the experiment. RNA was isolated at 4 h intervals and subjected to RT-qPCR. All graphs represent fold changes in expression at various time points relative to expression at the time of Dex,VPA addition and are the summary of of 3–6 independent biological replicates. Curve-fitting data from the cells exposed to VPA is indicated by the solid line. Curves derived from analysis of cells exposed to Dex alone (Fig. 3) are shown with a dotted line. Error bars represent standard error of the mean (SEM). Pound signs (#) represent significant changes between cells treated with Dex and cells treated with the combination of Dex and VPA. # – $p < 0.05$, ## – $p < 0.01$, ### – $p < 0.001$. (F) Dex-induced NIH/3T3 cells were treated with VPA before and after Dex removal and harvested at 1, 8, 24 and 48h. UT

denotes untreated cells harvested at the time of Dex addition. Whole cell lysates were subjected to SDS-PAGE and immunoblotting with antibodies against H3K27Ac, acetylated histone H4, acetylated histone H3, and total histone H3.

Figure 5. Addition of Valproic Acid at CT12 causes phase shifts in expression of genes encoding TFs belonging to both limbs of the circadian transcriptional feedback loop. NIH/3T3 cells were synchronized to a circadian cycle as described previously. At CT12 (12 hours from when Dex removal), the cells were treated with VPA (5mM). The cells were then harvested every 4h until 48 h. All graphs represent fold changes in expression at various time points relative to expression at the time of Dex,VPA addition and are the summary of 4–7 independent biological replicates. Curve-fitting data from the cells exposed to VPA is indicated by the solid line. Curves derived from analysis of cells treated with Dex alone (Fig. 3) are shown with a dotted line. Error bars represent standard error of the mean (SEM). # – $p < 0.05$, ## – $p < 0.01$, ### – $p < 0.001$. Pound signs (#) represent significant changes between cells treated with Dex and cells treated with the combination of Dex and VPA.

TABLE 1 -

PCR Primers used in the study

Gene	Forward primer	Reverse primer
<i>Tgm2</i>	GACAATGTGGAGGAGGGATCT	CTCTAGGCTGAGACGGTACAG
<i>Sgk1</i>	AGGAGCCGGAGCTTATGAAC	AGTGAAAGTCGGAGGGTTTGG
<i>Ampd3</i>	AAGATGATCCGGTCGCAGTC	CCAGGCTTAGAAGTAGTCCG
<i>Per 1</i> (exon-exon)	CTGGGGACCAGGTCATTAAGT	CACACACGCCATCACATCAA
<i>Per1</i> (exon-intron)	CCAGCCTGGCTGATGACACTGA	CACTCGTACACACCTCTTGCTGCTC
<i>Per2</i>	CAACATCCCATCCCGGAAGG	GGTGAGCACTGACCTCTGTG
<i>Nr1d1 (Reverba)</i>	GCTCAGCGTCATAATGAAGCG	GGGCCGAATATACGTGGGT
<i>Cry1</i>	CAGACTCTCGTCAGCAAGATG	CAAACGTGTAAGTGCCTCAGT
<i>Cry2</i>	TGGGCATCAACCGATGGAG	CCCATTCTTGAACAGCCTTG
<i>Bmal1</i> (exon-exon)	GGCTGTTTCAGCACATGAAAAC	GCTGCCCTGAGAATTAGGTGTT
<i>Bmal1</i> (exon-intron)	CCTGCACTCGCACATGGTTCCA	CCCCACAGGTTGAGAATCGCTACAT

TABLE 2 -
Baselines and amplitudes of oscillatory circadian transcription factor gene expression
under conditions of Dex alone or cotreatment with VPA.

Shaded values are derived from experiments in which VPA was present at CT0.

<i>BASELINE</i>					
Gene	Dex	VPA, Dex	<i>p</i> value	CT12 VPA, Dex	<i>p</i> value
Per2	1.325 ± 0.05683	1.399 ± 0.1154	0.5331	1.083 ± 0.0794	0.0474
Reverba	1.475 ± 0.07423	1.208 ± 0.0911	0.0388	1.799 ± 0.1499	0.0745
Cry1	1.034 ± 0.02368	1.423 ± 0.0521	<0.0001	1.326 ± 0.0634	<0.0001
Cry2	1.260 ± 0.04086	2.076 ± 0.1280	<0.0001	2.004 ± 0.1212	<0.0001
Bmal1	1.049 ± 0.05459	0.610 ± 0.0423	0.0003	1.019 ± 0.1583	0.8161
<i>AMPLITUDE</i>					
Gene	Dex	VPA, Dex	<i>p</i> value	CT12 VPA, Dex	<i>p</i> value
Per2	1.028 ± 0.08166	1.166 ± 0.1659	0.2704	0.357 ± 0.1147	0.0002
Reverba	0.394 ± 0.09741	0.384 ± 0.1194	0.6009	1.032 ± 0.2190	0.0120
Cry1	0.327 ± 0.03646	0.230 ± 0.0764	0.2170	0.435 ± 0.0973	0.2213
Cry2	0.282 ± 0.06032	0.502 ± 0.1925	0.1508	0.201 ± 0.1665	0.3714
Bmal1	0.463 ± 0.07271	0.337 ± 0.0660	0.8602	1.018 ± 0.2274	0.0036

Baseline values are averages (± standard error) over all replicates expressed in fold change units relative to untreated cells at the time of Dex treatment. Amplitude values are represented as the average change (± standard error) in expression (in fold change units) at peaks and troughs relative to the corresponding baseline. The *p* values were derived through comparison of the curve-fitting for cotreatment and Dex treatment alone using the extra sum of squares test.

TABLE 3 -
Times of Peaks and Troughs of circadian gene expression: Dex alone versus Dex plus VPA added at CT0

<i>VPA at CT0</i>				
Clock gene	Time of peak (h), Dex alone	Time of peak (h), VPA, Dex	Time of troughs (h), Dex alone	Time of troughs (h), VPA, Dex
Per2	3.6, 27.6	3.4, 27.4	15.8, 29.8	15.5, 29.5
Reverba	1.0, 25.0	1.1, 25.1	13.1, 37.1	13.0, 37.0
Cry1	5.9, 29.9	6.3, 28.3	17.7, 41.7	18.3, 42.3
Cry2	4.1, 28.1	4.6, 28.6	16.2, 40.2	16.1, 40.1
Bmal1	13.8, 37.8	20.0, 44.0	1.7, 25.7	7.9, 31.9

TABLE 4 -
Times of Peaks and Troughs of circadian gene expression: Dex alone versus Dex plus VPA added at CT12

<i>VPA at CT12</i>				
Clock gene	Time of peak (h), Dex alone	Time of peak (h), VPA, Dex	Time of troughs (h), Dex alone	Time of troughs (h), VPA, Dex
Per2	27.6	32.5	15.8, 29.8	20.7, 44.7
Reverba	25.0	28.6	13.1, 37.1	16.4, 40.4
Cry1	5.9, 29.9	18.4, 42.4	17.7	30.5
Cry2	4.1, 28.1	--	16.2, 40.2	--
Bmal1	13.8, 37.8	17.1, 42.1	25.7	28.9