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

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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.

1. 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 is associated with a variety of pathological states that include perturbations of metabolism, immune function, sleep, and fertility, among others (Sen and Sellix, 2016; Castanon-Cervantes et al., 2010; McHill and Wright Jr., 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 and Gasiewicz, 2006; Xu et al., 2010) and drugs (Li and Levi, 2007; Li et al., 2002; Johansson et al., 2011; Koyanagi and Ohdo, 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 > 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.-erbα 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).

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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 and Weitz, 2014). Finally, Rev.-erbα (encoded by the *Nr1d1* gene) has been found to recruit the NCoR/KDAC3 complex to repress *Bmal1* transcription (Yin and Lazar, 2005). In mouse liver, oscillatory association of Rev.-erbα 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 and Zaidman, 1988; Nanau and Neuman, 2013; Chang and Abbott, 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 and Sassone-Corsi, 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.

2. Materials and methods

2.1. 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 (MEMα) 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 (Clone D1H2) was obtained from Cell Signaling. The secondary goat anti-rabbit antibody (111–035-144) was purchased from Jackson Immunoresearch.

2.2. 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 (5 mM) 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,

Table 1
PCR Primers used in the study.

Gene	Forward primer	Reverse primer
Tgm2	GACAATGTGGAGGAGGGATCT	CTCTAGGCTGAGACGGTACAG
Sgk1	AGGAGCCGGAGCTTATGAAC	AGTGAAAGTCGGAGGGTTTGG
Ampd3	AAGATGATCCGGTCGCAGTC	CCAGGCTTAGAAGTAGCTCCG
Per 1 (exon-exon)	CTGGGGACCAGGTCATTAAGT	CACACACGCCATCACATCAA
Per1 (exon-intron)	CCAGCCTGGCTGATGACACTGA	CACTCGTACACACCTCTTGCTGCTC
Per2	CAACATCCCATCCCGGAAGG	GGTGAGCACTGACCTCTGTG
Nr1d1 (Reverbα)	GCTCAGCGTCATAATGAAGCG	GGGCCGAATATACGTGGGT
Cry1	CAGACTCTCGTCAGCAAGATG	CAAACGTGTAAGTGCCTCAGT
Cry2	TGGGCATCAACCGATGGAG	CCCATTCCTTGAACAGCCTTG
Bmal1 (exon-exon)	GGCTGTTCAGCACATGAAAAC	GCTGCCCTGAGAATTAGGTGTT
Bmal1 (exon-intron)	CCTGCACTCGCACATGGTTCCA	CCCCACAGGTTGAGAATCGCTACAT