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The mood stabilizer valproic acid opposes the effects of dopamine on circadian rhythms

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ABSTRACT

Endogenous circadian (~24 h) clocks regulate key physiological and cognitive processes via rhythmic expression of clock genes. The main circadian pacemaker is the hypothalamic suprachiasmatic nucleus (SCN). Mood disorders, including bipolar disorder (BD), are commonly associated with disturbed circadian rhythms. Dopamine (DA) contributes to mania in BD and has direct impact on clock gene expression. Therefore, we hypothesized that high levels of DA during episodes of mania contribute to disturbed circadian rhythms in BD. The mood stabilizer valproic acid (VPA) also affects circadian rhythms. Thus, we further hypothesized that VPA normalizes circadian disturbances caused by elevated levels of DA. To test these hypotheses, we examined locomotor rhythms and circadian gene cycling in mice with reduced expression of the dopamine transporter (DAT-KD mice), which results in elevated DA levels and mania-like behavior. We found that elevated DA signaling lengthened the circadian period of behavioral rhythms in DAT-KD mice and clock gene expression rhythms in SCN explants. In contrast, we found that VPA shortened circadian period of behavioral rhythms in DAT-KD mice and clock gene expression rhythms in SCN explants, hippocampal cell lines, and human fibroblasts from BD patients. Thus, DA and VPA have opposing effects on circadian period. To test whether the impact of VPA on circadian rhythms contributes to its behavioral effects, we fed VPA to *DAT*-deficient *Drosophila* with and without functioning circadian clocks. Consistent with our hypothesis, we found that VPA had potent activity-suppressing effects in hyperactive *DAT*-deficient flies with intact circadian clocks. However, these effects were attenuated in *DAT*-deficient flies in which circadian clocks were disrupted, suggesting that VPA functions partly through the circadian clock to suppress activity. Here, we provide *in vivo* and *in vitro* evidence across species that elevated DA signaling lengthens the circadian period, an effect remediated by VPA treatment. Hence, VPA may exert beneficial effects on mood by normalizing lengthened circadian rhythm period in subjects with elevated DA resulting from reduced DAT.

1. Introduction

As a result of regularly recurring daily cycles of sun exposure caused by earth's rotation, most living organisms have evolved circadian (ca. 24 h) clocks to anticipate daily environmental changes and orchestrate daily temporal programs of physiology and behavior. Within cells, autoregulatory clock proteins generate ca. 24 h rhythms by transcriptional-translational negative feedback loops. Core clock genes include *Bmal1*, *Clock*, *Period* (*Per1/2*) and *Cryptochrome* (*Cry1/2*). BMAL1 and CLOCK proteins heterodimerize and induce *Per* and *Cry* gene expression. Later in the day, PER and CRY proteins inhibit BMAL1/CLOCK and thereby their own transcription, thus completing the circadian cycle (Koike et al., 2012). In mammals, the master circadian pacemaker is located in the

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suprachiasmatic nucleus (SCN), in the hypothalamus, but most other tissues also harbor circadian clocks (Albrecht, 2012) which regulate the timing of key processes involved in metabolism, physiology, behavior, and mood regulation. These processes may in turn feedback to impact clock function as well. For example, disruption of circadian rhythms is a hallmark of most neuropsychiatric disorders, including bipolar disorder (BD) (Landgraf et al., 2014a; McCarthy and Welsh, 2012; Wulff et al., 2010), a disabling condition characterized by episodes of depression and mania. The mechanisms by which circadian and mood-regulating processes influence each other are unclear (Landgraf et al., 2014b). In the case of BD, however, manic behavior is associated with elevated levels of dopamine (DA) (van Enkhuizen et al., 2015), a neurotransmitter that also impacts circadian rhythms. For example, timed treatment with a D1 DA receptor agonist shifts circadian phase of fetal hamsters (Viswanathan and Davis, 1997; Viswanathan et al., 1994), possibly due to activation of D1 receptors in the mammalian SCN (Rivkees and Lachowicz, 1997; Weiner et al., 1991). DA also regulates core clock components in retina and striatum (Hood et al., 2010; Imbesi et al., 2009; Yujnovsky et al., 2006). Thus, DA may be crucially involved in both manic behavior and disrupted circadian rhythms in BD.

One possible reason for increased DA levels in BD patients is lower expression of the dopamine transporter (DAT) that removes DA from synaptic clefts. Variants of the *DAT* gene (SLC6A3) have been associated with BD and reduced DAT expression (Greenwood et al., 2001; Greenwood et al., 2006; Kelsoe et al., 1996). These associations are consistent with lower DAT levels observed in BD patients and thus elevated synaptic DA (Amsterdam and Newberg, 2007). Furthermore, in mice, knockout of DAT ($DAT^{-/-}$) increases DA levels and activity in new environments (Giros et al., 1996), and causes deficits in prepulse inhibition (Ralph et al., 2001). However, behavioral analysis of $DAT^{-/-}$ mice is complicated by poor physical state (Bosse et al., 1997). Mice with constitutive genetic knockdown of DAT (DAT-KD) express 10% of wild-type (WT) DAT levels and also show high DA levels. They display mania-like behaviors such as hyperactivity in a novel environment (Zhuang et al., 2001), replicating hyper-exploration and increased risk-taking behavior of manic BD patients (Perry et al., 2009; van Enkhuizen et al., 2014; Young et al., 2011b) without concomitant developmental defect. For these reasons, DAT-KD mice have been used as a model for mania. In BD patients, valproic acid (VPA) is used as a mood stabilizer, and particularly as an anti-manic agent. Interestingly, observations that VPA also attenuates the hyper-exploration of DAT-KD mice without affecting their WT littermates (van Enkhuizen et al., 2013) provided predictive validity for this model of BD mania (Young et al., 2011a).

Since DA regulates circadian clocks, we hypothesized that elevated DA levels contribute to circadian disturbances in manic BD patients. We tested this hypothesis in DAT-KD mice and cultured SCN explants treated with a D1 receptor agonist and found that increased DA signaling lengthens the period of circadian rhythms. Furthermore, we showed that VPA shortens circadian period, both *in vitro* and *in vivo*, and that the effects of VPA on behavior are less pronounced in the absence of a circadian clock in *DAT*-deficient *Drosophila melanogaster*. These data suggest that the mood-stabilizing properties of VPA in manic BD patients might be partly based on reversing the effects of elevated DA on circadian period.

2. Materials and methods

2.1. Animals

Mouse: All mice used for experiments were on a C57BL/6J genetic background. All mice used for behavioral assays were 19–21 week old male WT, DAT-$KD^{+/-}$, and DAT-$KD^{-/-}$ littermates (Zhuang et al., 2001). For organotypic SCN cultures we used 8–12 week old male $mPer2^{Luciferase}$ (PER2::LUC) mice. In PER2::LUC knockin mice, the circadian clock gene *Period2* (*Per2*) is replaced by homologous recombination with a construct incorporating the firefly luciferase (*Luc*) gene in tandem with WT *Per2*, such that a bioluminescent PER2::LUC fusion protein is expressed under control of all *Per2* regulatory elements (Yoo et al., 2004). In our mice, the reporter construct also incorporates an SV40 polyadenylation site to enhance expression levels (Welsh et al., 2004). Unless otherwise stated, mice were maintained in LD 12:12 cycles (12 h light, 12 h dark) and had *ad libitum* access to standard rodent chow and water. The time of "lights on" is defined as *Zeitgeber* time 0. We attempted to minimize the number of animals used and animal pain and distress. Mouse studies were conducted in accordance with regulations of the Institutional Animal Care and Use Committee at University of California, San Diego.

Drosophila: per^{01} mutant flies were obtained from Dr. Amita Sehgal (University of Pennsylvania). DAT^{fmn} mutant flies were obtained from Dr. Kazuhiko Kume (Kumamoto University). All mutants were outcrossed 4–5 times into a w^{1118} *iso31* genetic background and maintained at room temperature (20–22 °C) on standard cornmeal media with yeast.

2.2. Mouse behavioral assays

WT, DAT-$KD^{+/-}$, and DAT-$KD^{-/-}$ littermate mice were singly housed in running wheel-equipped cages, and locomotor activity was monitored for 28 weeks under various lighting conditions, with or without VPA in chow (see Fig. S1 for schedule). During the first three weeks of LD (LD1), light levels were 800 lux. For subsequent constant light (LL) and LD (LD2 and LD3) experiments, light levels were reduced to 300 lux. Light pulses (1 h, 500 lux) were given individually to mice two hours after behavioral activity onset (circadian time 14, CT14). During constant darkness (DD) experiments, daily health checks of mice were done at irregular times and under dim red light. VPA was administered in custom produced rodent chow (Harlan Teklab, USA). VPA chow contained 15 g VPA/kg chow and was otherwise identical to standard rodent chow. This dose was chosen based on previous work in which clinically relevant VPA serum concentrations (~70 μg/ml) were achieved in DAT-KD mice that attenuated their mania-like behaviors (van Enkhuizen et al., 2013). Wheel-running activity and phase shifts in response to light were analyzed using ClockLab software (Actimetrics, USA). The first 3–5 days of each new lighting condition were excluded from analysis. The same days were analyzed for each animal.

2.3. Drosophila behavioral assays

Individual 2–6 day old male flies were placed in 5 mm × 65 mm glass tubes containing 5% sucrose and 2% agarose at one end as a food source. Flies were then entrained to a LD 12:12 cycle for 2 days at 25 °C before measuring rest/activity patterns under the same conditions using the *Drosophila* Activity Monitoring System (Trikinetics, USA). Rest was defined as 5 min of sustained inactivity using a custom Matlab (Mathworks, USA) script. During drug treatment, activity and rest were recorded 24–48 h on standard food supplemented with 2.5 mM VPA (Sigma, USA). Animals that died within 2 days of drug exposure were excluded from analysis.

2.4. Cell and organotypic tissue culture

2.4.1. Hippocampal cell culture

Immortalized mouse mHippoE-14 hippocampal cell lines (Gingerich et al., 2010) were obtained commercially (Cedarlane,

USA). Cells were transduced with a lentiviral vector containing the bioluminescent circadian reporter gene *Per2*::*Luc* and a blasticidin resistance gene (Liu et al., 2007). Cells were selected with blasticidin (10 μg/ml) to obtain cells that stably express rhythmic *Per2*::*Luc*. Cells were plated in 35 mm dishes and grown to 70% confluence in standard culture medium [DMEM with 10% fetal bovine serum (FBS), 2 mM glutamine, and 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml Fungizone® Antimycotic (all purchased from Gibco)]. Cells were pre-treated with either vehicle or VPA (Tocris, USA) at a therapeutic concentration (1 mM) for 48 h, modeling chronic therapeutic exposure to VPA. Prior to rhythm recording, cells were exposed to 100 nM dexamethasone (Tocris, USA) for 2 h to synchronize rhythms, immediately followed by change to medium with or without 1 mM VPA. Bioluminescence rhythm measurements were then started immediately and lasted 5–7 days.

2.4.2. Human fibroblast cultures

Skin fibroblasts were obtained from BD (type 1) patients and control subjects as described previously (McCarthy et al., 2013). Cells were transduced with a lentiviral vector containing *Per2*::*Luc* and grown to 70% confluence in standard culture medium. As for neuronal cell lines, fibroblasts were pre-treated with VPA (1 mM) or vehicle for 48 h, followed immediately by medium change with fresh drug/vehicle and longitudinal measurement of rhythms.

2.4.3. SCN cultures

Mice were anesthetized with isoflurane and killed by cervical dislocation. Brains were kept at 4 °C. 300 μm brain slices were prepared with a vibratome (Leica, USA) and the SCN was cut out. Tissues were immediately transferred to tissue culture inserts (EMD Millipore, USA) and cultured in 35 mm petri dishes containing 1 ml of culture medium [high glucose DMEM (Mediatech, Manassas, VA, USA), 4 mM sodium carbonate, 10 mM HEPES, 52 U/ml penicillin, 52 μg/ml streptomycin, 4 mM L-glutamine, 2% B-27 (GIBCO, Grand Island, NY, USA), 0.1 mM luciferin (BioSynth, Itasca, IL, USA)].

2.5. Bioluminescence measurements

Measurements were done at 10 min intervals using a LumiCycle luminometer (Actimetrics, USA) that was placed inside a 37 °C incubator without CO_2. Period was determined over 5–7 days by fitting a damped sine wave [Sin fit (Damped)] to 24 h running average baseline-subtracted data using LumiCycle Analysis software (Actimetrics, USA). The first day of measurement was excluded from analyses.

2.6. Data analysis

Statistical analysis was carried out with GraphPad Prism (GraphPad Software, USA). Statistical tests, F-values, degrees of freedom, and p-values for each experiment are indicated in the figure legends.

3. Results

3.1. DAT-KD mice exhibited chronically increased locomotor activity in running wheels

DAT-KD mice were previously reported to exhibit increased spontaneous locomotor activity when exposed to a new environment (Giros et al., 1996; van Enkhuizen et al., 2013). Here we measured running-wheel behavior over the course of seven months and showed that DAT-KD mice display chronic hyperlocomotion in a familiar environment. Comparing WT, DAT-KD$^{+/-}$, and DAT-KD$^{-/-}$ littermate mice, we found that genotype was associated with the total activity of the mice under 12:12 LD and DD conditions (Fig. 1A, B, Fig. S2). In 12:12 LD, the genotype predicted the duration of the daily active phase (Fig. 1C). Although no significant interaction between genotype and time (LD1-3) was found, post hoc tests suggested that effects became more pronounced over time. While total activity decreased over time in WT mice, DAT-KD$^{+/-}$ and DAT-KD$^{-/-}$ mice remained highly active in LD2 and LD3 (Fig. 1B, Fig. S2). Moreover, in 12:12 LD, DAT-KD$^{-/-}$ mice displayed a very pronounced lengthening of their daily active phases in the final stage of the experiment (LD3) compared to WT and DAT-KD$^{+/-}$ mice (Fig. 1C).

3.2. DAT-KD$^{-/-}$ mice exhibited free-running locomotor activity rhythms with long circadian periods

Mice were entrained to 12:12 LD conditions for three weeks and then transferred to DD to monitor the endogenous SCN-driven ("free-running") locomotor activity rhythm. Over the next four weeks, DAT-KD$^{-/-}$ mice exhibited a significantly longer circadian period compared to WT and DAT-KD$^{+/-}$ littermate mice (Fig. 2A, B). In subsequent exposure to LL, DAT-KD$^{+/-}$ and DAT-KD$^{-/-}$ mice continued to show a tendency for longer free-running circadian periods compared to WT (Fig. S3).

3.3. DAT-KD$^{-/-}$ mice exhibited larger behavioral rhythm phase-shifting responses to light

To examine behavioral rhythm resetting responses to light, WT, DAT-KD$^{+/-}$, and DAT-KD$^{-/-}$ littermate mice were kept in constant darkness for one week and then exposed to a 1 h light stimulus at CT14, a time expected to elicit a phase delay of activity rhythms. Phase delays were significantly greater in DAT-KD$^{-/-}$ compared to the WT and DAT-KD$^{+/-}$ mice (Fig. 2C, D).

3.4. Elevated dopamine signaling lengthened circadian period by acute effects on D1 receptors in SCN

The SCN is the primary circadian pacemaker determining the period of circadian locomotor activity rhythms (Ralph et al., 1990). DAT-KD mice are characterized by elevated DA levels (Zhuang et al., 2001), and the SCN expresses D1 receptors. In order to test whether the long circadian period we observed in DAT-KD$^{-/-}$ mice could be explained by long-lasting effects of increased DA input to the SCN (e.g., during development), we cultured SCN explants from WT, DAT-KD$^{+/-}$, and DAT-KD$^{-/-}$ littermate mice that carried the PER2::LUC circadian reporter gene and tested the intrinsic SCN circadian period *in vitro*. We found no significant differences among the three genotypes in the period, amplitude, or phase of circadian PER2::LUC rhythms in SCN explants, suggesting that DAT knockdown has no sustained effect on SCN period in the absence of DA afferent input (Fig. 3A, Fig. S4). To test whether increased DA signaling acutely increases SCN circadian period, we treated WT SCN explants with a selective D1/D5 receptor agonist (50 μM SKF 38393). Activation of DA receptors lengthened circadian period of the SCN explants (Fig. 3B). Importantly, simultaneous treatment with a selective D1/D5 receptor antagonist (10 μM SKF 83566) blocked the period-lengthening effect of the agonist, showing that the effect is specific to DA receptor activation. Together, these results suggest that the sustained high DA levels in DAT-KD$^{-/-}$ mice increase circadian free-running period by action on DA receptors in the SCN, but that the period normalizes rapidly in the absence of sustained high DA afferent input.

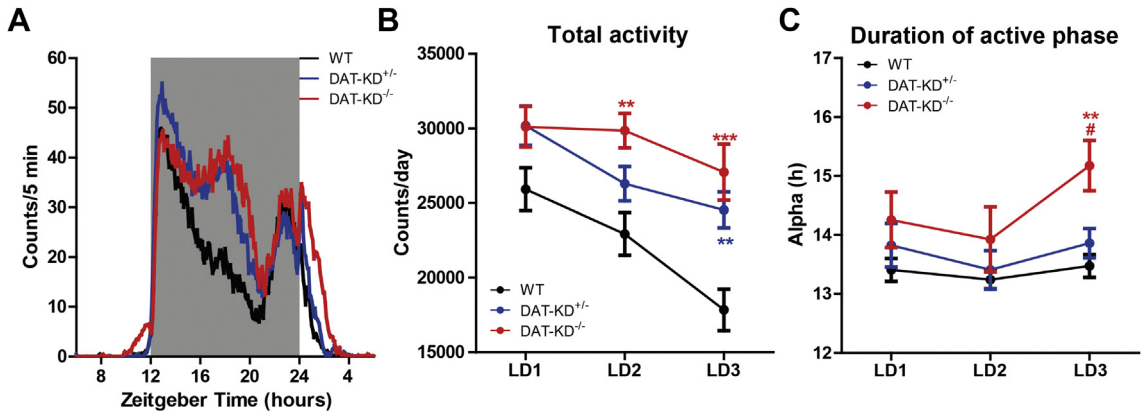

Fig. 1. Locomotor activity of DAT-KD mice is chronically increased in running wheels. (A) Activity profiles during light/dark cycle (LD3). Gray area represents dark phase. The mean of 20 successive days was calculated from all animals for each 5 min interval. (B) DAT-KD$^{+/-}$ and DAT$^{-/-}$ mice exhibit increased levels of activity (2-way repeated measurement ANOVA. Interaction $F_{4,50} = 1.85$, $p = 0.1344$; Genotype $F_{2,50} = 10.82$, $p = 0.0004$; Time $F_{2,50} = 20.29$, $p < 0.0001$). (C) In the LD3 segment, the activity offset of DAT$^{-/-}$ mice was delayed, which led to a prolonged duration of daily active phase (Alpha) (2-way repeated measurement ANOVA. Interaction $F_{4,50} = 1.73$, $p = 0.1586$; Genotype $F_{2,50} = 3.75$, $p = 0.0377$; Time $F_{2,50} = 5.90$, $p = 0.005$). Data are shown as mean ± SEM; post hoc: $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ comparing to WT, $^{\#}p \leq 0.05$ comparing to DAT-KD$^{+/-}$ (2-way repeated measure ANOVA with Bonferroni post-test comparing all groups with each other); n = 9–10.

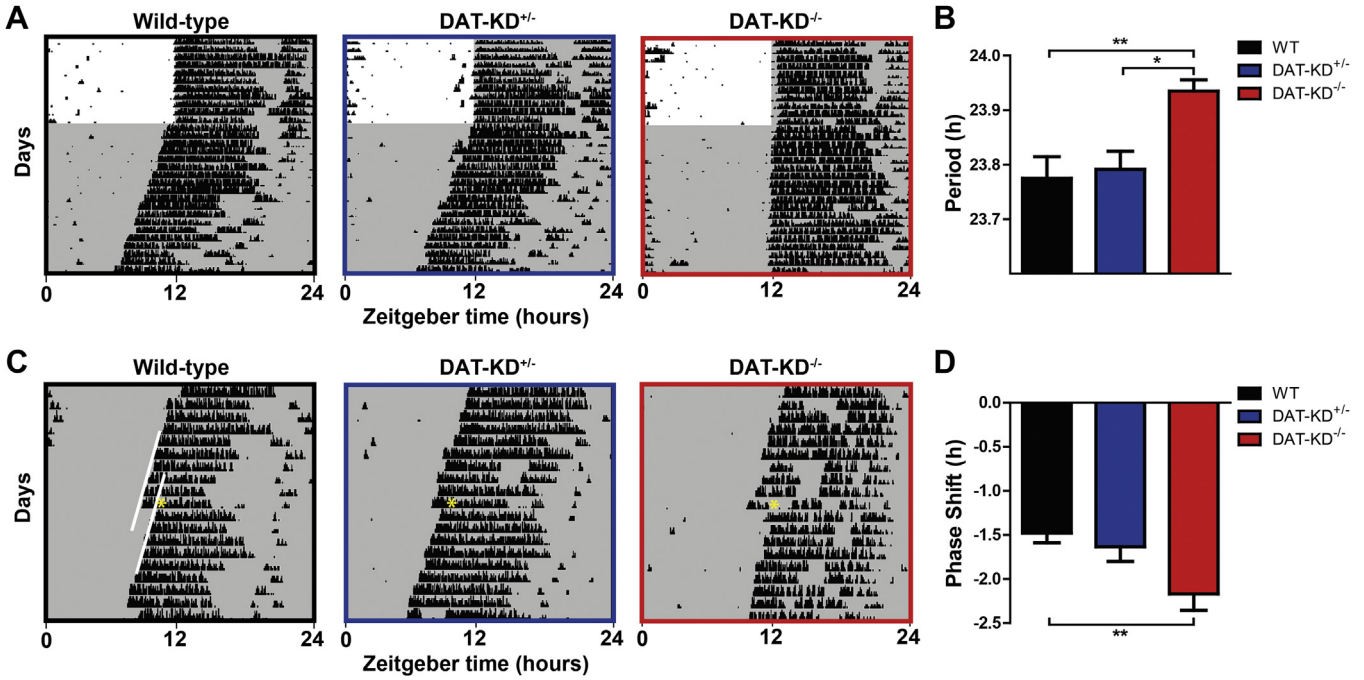

Fig. 2. DAT-KD$^{-/-}$ mice exhibit longer free-running periods and increased sensitivity to light pulses in constant darkness. (A) Representative actograms showing wheel-running activity in constant darkness after prior entrainment in light/dark. Gray areas represent darkness. (B) DAT-KD$^{-/-}$ mice exhibit longer free-running periods in constant darkness [$F_{2,27} = 7.504$]. (C) Representative actograms showing phase shifts of wheel-running activity in constant darkness in response to a light pulse (yellow asterisk) given at CT14. For WT, white lines illustrate the phase shift of activity onset. (D) Compared to WT, DAT-KD$^{-/-}$ mice exhibit more pronounced phase delays in response to the light pulse [$F_{2,27} = 5.221$]. Data are shown as mean ± SEM; post hoc: $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ (1-way ANOVA with Bonferroni post-test comparing all groups with each other); F-values with degree of freedom provided in squared brackets; n = 10. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.)

3.5. Valproic acid shortened circadian period of mouse behavior, SCN explants, and hippocampal neurons

To test the effect of VPA on free-running circadian locomotor rhythms, we chronically treated WT, DAT-KD$^{+/-}$, and DAT-KD$^{-/-}$ mice in DD with chow containing 1.5% VPA for five weeks, a treatment known to remediate hyperactivity in DAT-KD mice (van Enkhuizen et al., 2013). We found that VPA shortened the circadian period of behavioral rhythms in all mice, regardless of genotype (Fig. 4A, B). Notably, when the VPA treatment was discontinued, rhythms reverted to a longer period in all genotypes (2-way repeated measurement ANOVA, VPA treatment: $p \leq 0.001$). To test whether VPA targets the central clock directly, we cultured SCN explants from PER2::LUC mice and treated them with different concentrations of VPA. Whereas 1 mM VPA had no effect on circadian period in SCN cultures, 10 mM VPA significantly shortened the period (Fig. 4C). As for *in vivo*, when the VPA treatment was discontinued, rhythms reverted to a longer period. The VPA concentration required to affect the SCN clock *in vitro* is higher than therapeutic concentrations used in humans. However, *in vivo* effects on mouse behavioral activity levels and circadian rhythms do appear at lower concentrations, leading us to consider whether

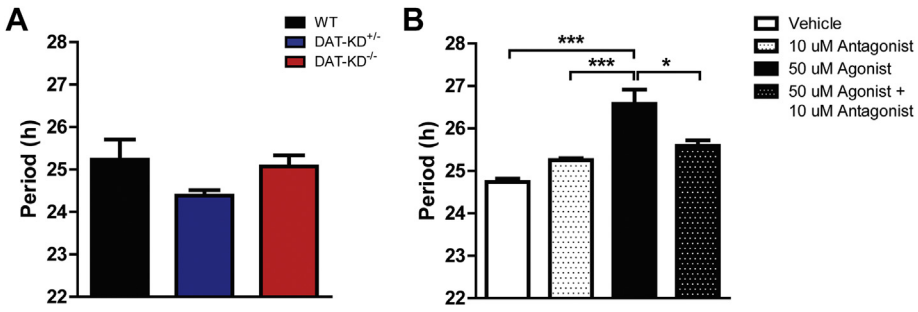

Fig. 3. Dopamine lengthens the circadian period of SCN explants. (A) When cultured in the absence of DA receptor agonists, cultured SCN explants of WT, DAT-KD$^{+/-}$, and DAT$^{-/-}$ mice show similar PER2::LUC rhythm periods. Data are shown as mean ± SEM; $F_{2,17} = 1.669$, post hoc: not significant (1-way ANOVA with Bonferroni post-test comparing all groups with each other); n = 6–7. (B) In the presence of a selective D1/D5 receptor agonist (SKF 38393), the PER2::LUC rhythm period of WT SCN explants is significantly longer. The additional administration of a selective D1/D5 antagonist (SKF 83566) attenuates the effects of the agonist. Data are shown as mean ± SEM; $F_{6,45} = 8.792$, post hoc: $^{*}p \leq 0.05$, $^{***}p \leq 0.001$ (1-way ANOVA with Bonferroni post-test comparing all groups with each other); n = 8–10.

non-SCN brain areas might be more sensitive to VPA and mediate the behavioral changes. Therefore, we also tested the effect of VPA on a mouse hippocampal cell line, as the hippocampus has been implicated in mood regulation (Campbell and Macqueen, 2004). Indeed, we found that 1 mM VPA significantly shortened the period of PER2::LUC rhythms in hippocampal neurons, demonstrating that VPA, at a therapeutic concentration, affects circadian clocks in cells derived from a brain region implicated in mood regulation (Fig. 4D).

3.6. Valproic acid shortened circadian period in fibroblasts from BD patients and controls

We next asked whether the effects of VPA we found in mouse behavior, SCN, and hippocampal neurons might be relevant for human cells. Since, skin fibroblasts from BD patients have longer circadian periods than fibroblasts from control subjects (McCarthy et al., 2013), we tested whether VPA can normalize the periods of these cells. We found that 1 mM VPA significantly shortened circadian period of *Per2-luc* rhythms in fibroblasts from either BD patients or healthy control subjects (2-way repeated measurement ANOVA, VPA treatment: $p \leq 0.001$) (Fig. 4E). Importantly, this effect altered circadian periods of BD fibroblasts to values similar to those of control cells. In summary, VPA shortens circadian period not only of behavioral, SCN, and hippocampal neuron rhythms in mice, but also of human fibroblasts, particularly from BD patients.

3.7. Suppression of behavioral activity in Drosophila was partly dependent on circadian clocks

Previous studies and our results showed that DA contributes to mania-like behavior and, in addition, lengthens the period of circadian rhythms in mice, whereas VPA reverses these effects (van Enkhuizen et al., 2013). These results led us to hypothesize that VPA may stabilize mood partly by adjusting circadian clocks. To test this hypothesis, we examined the behavioral effects of VPA in *DAT*-deficient *Drosophila melanogaster* (*DAT^{fmn}*) with and without functional circadian clocks. As previously demonstrated (Kume et al., 2005), *DAT^{fmn}* mutants had elevated locomotor activity, much like DAT-KD mice, and reduced duration of sustained rest (bouts of ≥5 min inactivity). We then abolished circadian rhythms in these animals by combining the *DAT^{fmn}* mutation with a null mutation in the *period* gene (*per^{01}*). As with *DAT^{fmn}* alone, *per^{01};DAT^{fmn}* double mutants exhibited increased total activity and reduced duration of sustained rest (Fig. 5A + B, Fig. S5). Interestingly, treatment with VPA reduced total activity in both *DAT^{fmn}* and *per^{01};DAT^{fmn}* flies (Fig. 5A), but the effect was less pronounced in the latter, which lacked a functional circadian clock. VPA also partly restored normal levels of sustained rest to clock-containing *DAT^{fmn}* flies, but not to clock-deficient *per^{01};DAT^{fmn}* flies (Fig. 5B). Importantly, at the concentration of VPA used for both sets of experiments the drug had no effect on activity or sustained rest of wild-type control animals. Thus collectively, our results suggest that antimanic effects of VPA may partly depend on functioning circadian clocks.

4. Discussion

We hypothesized that elevated DA levels change the period of circadian rhythms. To test this hypothesis, we measured circadian rhythms in locomotor activity of DAT-KD mice, an animal model of mania with chronically high extracellular DA levels. We found that DAT-KD$^{-/-}$ mice exhibited lengthened free-running circadian periods. Period lengthening was not present in SCN slice cultures, which lack DA afferent inputs. However, in the presence of a D1/D5 receptor agonist (SKF 38393) the period of SCN explants became significantly longer. Although we did not measure *in vivo* DA levels in the SCN, these findings suggest that all three genotypes have intrinsically similar SCN clocks, but that high DA input to the SCN in DAT-KD$^{-/-}$ mice lengthens circadian period *in vivo*. Moreover, VPA, a mood-stabilizing agent used in the treatment of BD, significantly attenuated the lengthened period of DAT-KD$^{-/-}$ mice and cells, suggesting that its therapeutic properties in BD could be related to its period-shortening effects.

Connections between DA signaling and circadian rhythms have been described previously. Suppression of DA decreases PER2 levels in the dorsal striatum of rats (Hood et al., 2010). Conversely, activation of D1 or D2 receptors increases *Per1* transcription in striatum and retina (Imbesi et al., 2009; Yujnovsky et al., 2006). Notably, *Per1* overexpression leads to longer circadian periods (Numano et al., 2006). Thus, elevated DA levels in DAT-KD$^{-/-}$ mice may lengthen circadian period by enhancing *Per1* expression. Additionally, an independent methamphetamine-sensitive circadian oscillator (MASCO) may interact with the SCN to alter behavioral rhythms of mice. Independent of the SCN and of circadian clock function, chronic methamphetamine administration induces activity rhythms with a period close to 24 h (Mohawk et al., 2009; Tataroglu et al., 2006). A recent study from Blum and colleagues indicates that the MASCO is a manifestation of a newly discovered dopaminergic oscillator (Blum et al., 2014). They have shown that in the absence of a functional molecular circadian clock, hyperdopaminergic DAT$^{-/-}$ mice have longer period ultradian rhythms, and increasing DA signaling can even evoke behavioral rhythms with periods up to 24 h and longer. Thus, in DAT-KD$^{-/-}$ mice, circadian clocks and the dopaminergic oscillator may interact to produce an overall

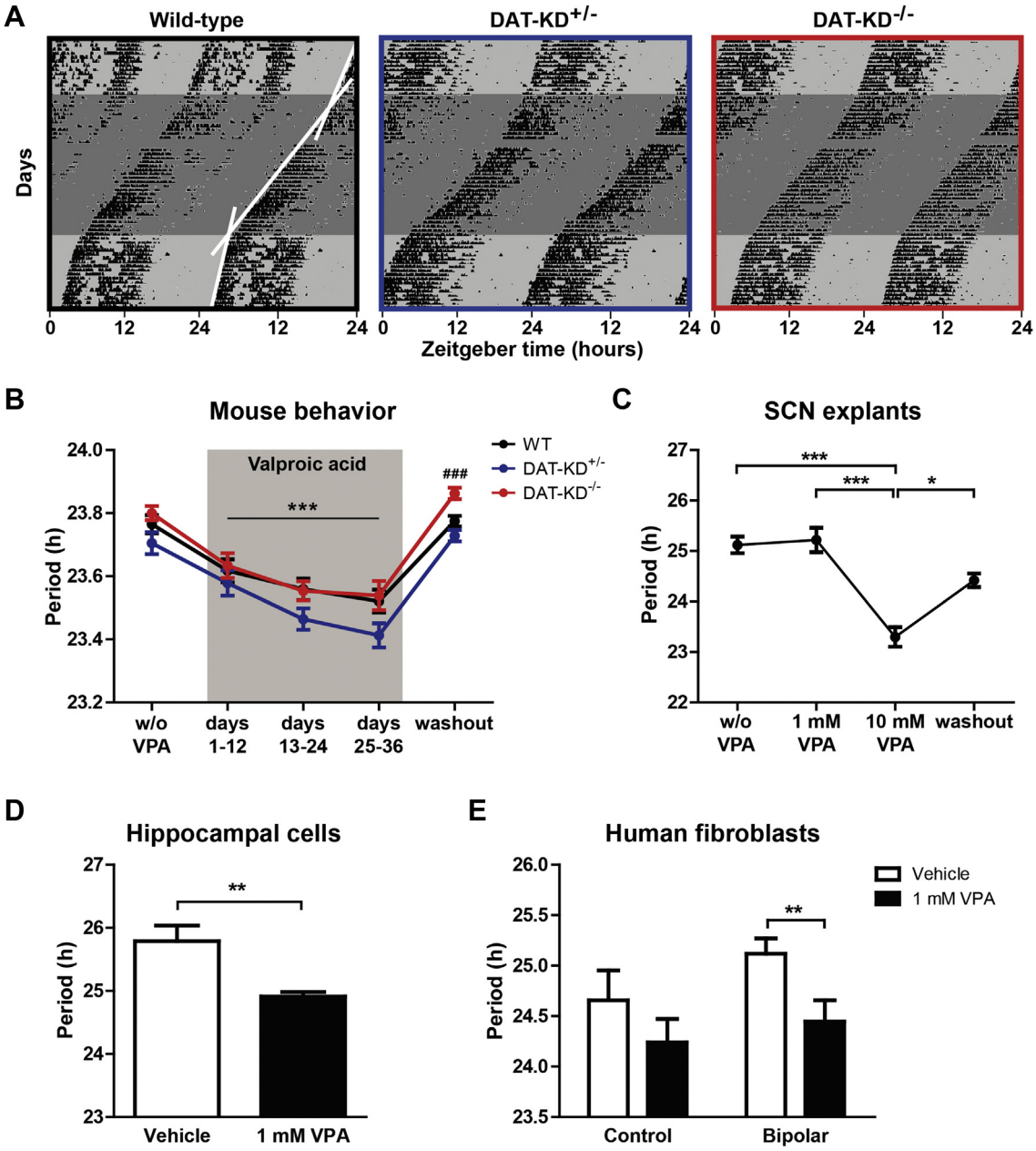

Fig. 4. VPA shortens the circadian period in mice, SCN explants, mouse hippocampal neurons, and human fibroblasts. (A) Representative actograms showing wheel-running activity in constant darkness prior to, during, and after administration of VPA. Dark regions indicate time of VPA treatment. For WT, white lines illustrate period changes. (B) VPA treatment (dark region) shortens the free-running period of wheel-running activity rhythms in WT, DAT-KD$^{+/-}$, and DAT$^{-/-}$ mice (2-way repeated measurement ANOVA. Interaction $F_{6,60} = 0.39$, $p = 0.8816$; Genotype $F_{2,60} = 4.04$, $p = 0.0335$; VPA $F_{3,60} = 46.24$, $^{***}p < 0.0001$). When the treatment is discontinued, the free-running behavior becomes significantly longer again (2-way repeated measurement ANOVA. Interaction $F_{2,20} = 1.58$, $p = 0.2304$; Genotype $F_{2,20} = 5.51$, $p = 0.0124$; VPA $F_{1,20} = 277.54$, $^{###}p < 0.0001$). Data are shown as mean ± SEM; n = 7–8. (C) 10 mM VPA shortens PER2::LUC period of WT SCN explants. Data are shown as mean ± SEM; $F_{3,12} = 19.73$, post hoc: $^{*}p \leq 0.05$, $^{***}p \leq 0.001$ (1-way repeated measure ANOVA with Bonferroni post-test comparing all groups with each other); n = 5. (D) 1 mM VPA shortens PER2::LUC rhythm period of cultured murine hippocampal cells. Data are shown as mean ± SEM; $^{**}p \leq 0.01$, $t_{14} = 3.424$ (Student's t-test); n = 8. (E) 1 mM VPA shortens PER2::LUC rhythm period of cultured human fibroblasts from BD patients and control subjects. Data are shown as mean ± SEM; post hoc: $^{*}p \leq 0.05$, $^{***}p \leq 0.001$ (2-way ANOVA with Bonferroni post-test: Interaction $F_{1,25} = 1.07$, $p = 0.3099$; VPA $F_{1,25} = 19.64$, $p = 0.0002$; Disorder $F_{1,25} = 1.29$, $p = 0.2674$); n = 13–14.

rhythmic output with a period longer than in WT mice.

Interestingly, many but not all studies of BD patients show delayed sleep onset (late chronotype) (Ahn et al., 2008; Mansour et al., 2005; Wood et al., 2009), which is associated with a long circadian period under free-running conditions (Brown et al., 2008; Duffy et al., 2001; Roenneberg and Merrow, 2000). The data from our present study show that high levels of DA lengthen circadian period, possibly explaining a portion of the circadian disturbances observed in BD patients. However, we have shown previously that cultured fibroblasts from BD patients, which lack DA inputs, also display longer period circadian rhythms compared to healthy subjects (McCarthy et al., 2013). Thus, the long period phenotype in BD could have multiple explanations, including heightened DA inputs, but also non-DA inputs or intrinsic aspects of cellular clock function. Further work is required to determine if these period altering mechanisms interact, and if so to what extent. Elevated DA levels presumably affect circadian rhythms preferentially in neurons receiving dopaminergic input. Thus, in addition to lengthening

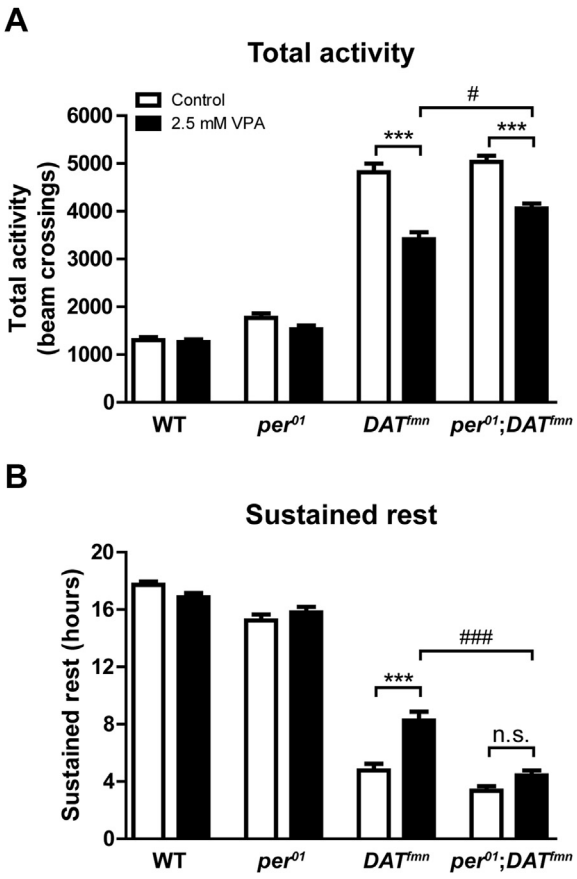

Fig. 5. In *Drosophila*, effects of VPA are partly dependent on circadian clocks. (A) Total daily locomotor activity in LD. Compared to WT and per^{01} controls, DAT^{fmn}, and per^{01};DAT^{fmn} flies show increased activity (each $p \leq 0.001$, not indicated in graph). 2.5 mM VPA partly reverses the hyperactivity phenotype, but is less effective in per^{01};DAT^{fmn} flies, which lack a functional circadian clock [$F_{7,335} = 154.4$]. (B) Compared to WT and per^{01} controls, hours of sustained rest (bouts of ≥5 min inactivity) are reduced in DAT^{fmn} and per^{01};DAT^{fmn} flies (each $p \leq 0.001$, not indicated in graph). 2.5 mM VPA increases sustained rest in clock-containing DAT^{fmn} flies, but not in clock-deficient per^{01};DAT^{fmn} flies [$F_{7,335} = 243.3$]. Data are shown as mean ± SEM; post hoc: $^{***}p \leq 0.001$ comparing control vs. 2.5 mM, $^{\#}p \leq 0.05$, $^{\#\#\#}p \leq 0.001$ comparing 2.5 mM/DAT^{fmn} vs. 2.5 mM/per^{01};DAT^{fmn} (1-way ANOVA with Bonferroni post-test comparing all groups with each other); F-value with degree of freedom provided in squared brackets; WT: n = 29–32, per^{01}: n = 32, DAT^{fmn}: n = 31–32, per^{01};DAT^{fmn}: n = 77–78.

circadian period in the SCN, increased DA may also provoke internal desynchrony among different brain regions involved in mood regulation. Circadian misalignment between brain regions could contribute to mood-related abnormalities (Landgraf et al., 2015; McCarthy and Welsh, 2012). Indeed, in DAT-KD$^{-/-}$ mice we did not find that longer periods of locomotor activity rhythms were correlated with hyperactivity (data not shown), which is consistent with the idea that DA-induced internal desynchrony of circadian rhythms within the brain (not necessarily accompanied by long period of SCN or behavioral rhythms) may contribute to mania-like behavior.

Because the mood stabilizer VPA alleviates hyperactivity in DAT-KD mice (van Enkhuizen et al., 2013), we tested whether it can shorten circadian period. Previous work has shown that VPA shifts the phase and increases amplitude of PER2 rhythms in cultured SCN explants and fibroblasts (Johansson et al., 2011). In hamsters, low concentrations of VPA had no effect on circadian period (Klemfuss and Kripke, 1995). However, in our study, higher VPA concentrations shortened free-running period of WT, DAT-KD$^{+/-}$, and DAT-KD$^{-/-}$ mice. We found that VPA also shortened the period of cultured WT SCN explants, thus demonstrating that VPA has a direct impact on the molecular clock in the SCN. However, relatively high concentrations (10 mM) of VPA were required for this effect, possibly because the SCN is characterized by unique network properties that make the SCN clock robust against perturbations that disrupt rhythms more readily in single cells (Buhr et al., 2010; Liu et al., 2007). And we also found that lower, therapeutic concentrations of VPA (1 mM) did shorten the circadian period of neurons derived from the mouse hippocampus, a brain region critically involved in behavior and mood regulation (Campbell and Macqueen, 2004). Importantly, we also found that VPA shortened circadian period in human cells, including cells of BD patients with long circadian periods. Thus, VPA at therapeutic concentrations could potentially restore normal cycling of circadian clocks in brain regions outside of the SCN, thereby restoring proper synchronization across brain regions, and this might explain part of its hyperactivity-attenuating effects in DAT-KD$^{-/-}$ mice (van Enkhuizen et al., 2013), and its therapeutic value in BD. Whether the effects of VPA on circadian period are potentially relevant for other symptoms of mania needs to be determined in future studies.

It is of interest to contrast the period-shortening effects of VPA to the period-lengthening effects of lithium, another mood stabilizer (Li et al., 2012). The opposing effects of these two drugs on circadian rhythms might seem to imply that their circadian effects are unrelated to their mood-stabilizing effects. However, closer scrutiny reveals complexities that confound such a simple interpretation. Although the therapeutic mechanism of lithium is unknown, inhibition of GSK3β is commonly postulated as a critical component. Unlike lithium and similar to VPA, selective GSK3β inhibitors shorten circadian period (Hirota et al., 2008), so if GSK3β inhibition is essential for mood stabilization, circadian effects of lithium or VPA could be incidental and "off target". But alternatively, the opposite effects of lithium and VPA on circadian period might still be clinically relevant but specific to different subpopulations of BD patients. Clinical response to mood stabilizers is not uniform: only about 1/3 of BD patients respond fully to lithium, and the clinical profiles that predict lithium vs. VPA response differ considerably (McCarthy et al., 2010). Accordingly, while many studies have reported a predisposition towards phase delay/long periods in BD, some have reported phase advance/short period in BD (Kripke et al., 1978; Salvatore et al., 2008) or other mood disorders (Lewy et al., 2006). Perhaps only a subset of patients with long period/phase delay benefit from the period-shortening effects of VPA, while a different subset with short period/phase advance benefit from lithium. Moreover, there are important differences in the clinical use of these two drugs. Lithium is both an anti-manic and anti-depressant drug, while VPA is effective predominantly in the manic phase (VA/DoD Bipolar Disorder Working Group, 2010). Thus, both trait and state characteristics of patients may determine whether period lengthening or shortening is therapeutic. Understanding the relationship of circadian period to mood could inform future "precision medicine" efforts that aim to better match a drug to an individual BD patient.

DAT-KD$^{-/-}$ mice showed more pronounced phase shifts of behavioral rhythms in response to light pulses than WT and DAT-KD$^{+/-}$ mice. Interestingly, several studies have reported that BD patients are more sensitive to light than control subjects (von Knorring, 1978). In BD patients, lower light intensities are needed to suppress melatonin, and high light levels lead to more pronounced melatonin suppression (Lewy et al., 1981, 1985; Nathan et al., 1999). Thus, increased responsiveness to light may further contribute to abnormal circadian rhythms in BD patients. Notably, increased sensitivity to light can be reduced with the mood stabilizers lithium and VPA (Hallam et al., 2005; Seggie et al., 1989).

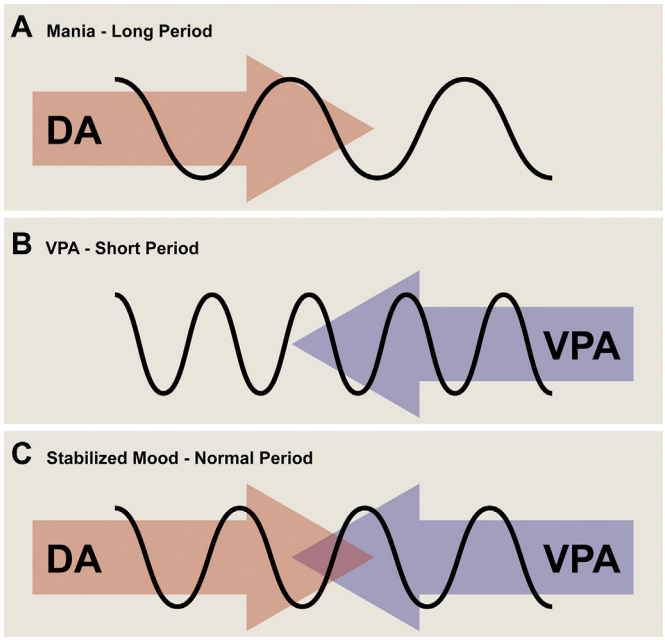

Fig. 6. Opposing effects of DA and VPA on circadian rhythms. (A) High levels of DA contribute to mania and lengthen the period of circadian rhythms. (B) VPA shortens the period of circadian rhythms. (C) In the presence of high dopamine levels, VPA adjusts circadian rhythms to a normal period.

Based on our results and findings from previous studies, we hypothesized that mood-stabilizing effects of VPA are partly dependent on its ability to normalize circadian rhythms. If this assumption is correct, effects of VPA should be less pronounced in the absence of circadian clocks. We therefore tested the effects of VPA on the behavior of *DAT*-deficient *Drosophila melanogaster* with and without functional circadian clocks. In accordance with our hypothesis, effects of VPA on daily activity levels and rest times were less pronounced in arrhythmic per^{01};DAT^{fmn} than in rhythmic DAT^{fmn} animals. However, the DAT mutation and VPA still had clear effects on mood-related behaviors of arrhythmic flies, indicating that these effects are not entirely dependent on circadian clocks. Mechanisms of action of VPA include blockade of voltage-dependent sodium channels and increase of gamma-aminobutyric acid (GABA), leading to an overall reduction of neuronal activity (Loscher, 2002). VPA has also been shown to inhibit GSK3β indirectly (Chen et al., 1999; De Sarno et al., 2002), and to block the activity of histone deacetylases (HDACs). Given the results of our study, in which VPA shortened period similarly in both neurons and fibroblasts (which lack DA and its receptors), the effects of VPA on circadian rhythms must be independent of DA receptor signaling. Thus, effects of DA and VPA on circadian period must be mediated through opposing effects on intracellular signaling pathways.

5. Conclusions

In summary, elevated DA levels contribute to mania-like behavior in mice and also lengthen periods of circadian rhythms (Fig. 6A). Circadian effects may be particularly pronounced in brain areas that receive high DA input, leading to circadian misalignment in the brain. In contrast, VPA attenuates mania-like behavior and shortens periods of circadian oscillations (Fig. 6B). Since VPA effects are less pronounced in animals lacking circadian rhythms, it is possible that normalization of lengthened circadian periods is a novel mechanism by which VPA regulates mood (Fig. 6C).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.neuropharm.2016.03.047.

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