Bipolar disorder (BD) is a severe psychiatric illness affecting ~1% of the world population. Valproate (VPA) and lithium, widely used for the treatment of BD, are not universally effective. These drugs have been shown to cause inositol depletion, but translating this observation to a specific therapeutic mechanism has been difficult, hampering the development of more effective therapies. We have shown previously in yeast that chronic VPA treatment induces the unfolded protein response due to increasing ceramide levels. To gain insight into the mechanisms activated during acute VPA treatment, we performed a genome-wide expression study in yeast treated with VPA for 30 min. We observed increased mRNA and protein levels of RSB1, which encodes an exporter of long chain bases dihydrosphingosine (DHS) and phytosphingosine (PHS), and further saw that VPA increased sensitivity of an rsb1Δ mutant to PHS, suggesting that VPA increases long chain base levels. Consistent with this, PHS levels were elevated in wild type and, to a greater extent, in rsb1Δ cells. Expression of ORM genes (negative regulators of PHS synthesis) and of fatty acid elongase genes FEN1 and SUIR4 were decreased, and expression of YOR1 (exporter of PHS-1P) and DPL1 (lyase that degrades DHS-1P and PHS-1P) was increased. These effects were more pronounced in medium lacking inositol, and were mirrored by inositol starvation of an ino1Δ mutant. These findings provide a metabolic explanation as to how VPA-mediated inositol depletion causes increased synthesis of PHS and further support the therapeutic relevance of inositol depletion as a bipolar disorder treatment.

Valproic acid (VPA)3 is a widely used drug for the treatment of bipolar disorder (BD), a devastating psychiatric illness that affects ~1% of the population (1). However, similar to other treatments for the disorder, the efficacy of the drug is not universal, and there is an urgent need to develop more effective therapies. A major obstacle to the identification of better drugs to treat BD is the lack of knowledge of the therapeutic mechanisms of action of the current drugs (2). Inositol depletion has been proposed to explain the therapeutic efficacy of BD drugs based on the finding that lithium causes inositol depletion by inhibiting inositol monophosphatase (IMPase) (3). This was hypothesized to result in decreased levels of inositol, an increase in inositol phosphates, and subsequent down-regulation of the phosphoinositide cycle. More recent studies show that VPA also decreases intracellular inositol levels in yeast and mammalian cells, not by inhibiting IMPase but by indirectly inhibiting myo-inositol-3-phosphate synthase, which catalyzes the rate-limiting step in the de novo synthesis of inositol (4–7). The finding that structurally dissimilar drugs inhibit inositol synthesis, albeit by affecting different enzymes, suggests that inositol depletion may be important for the therapeutic mechanism of action.

In yeast, VPA causes a decrease in intracellular inositol and a dramatic increase in expression of INO1, the gene encoding myo-inositol-3-phosphate synthase (5). Interestingly, the drug exerts different acute and chronic effects on INO1 expression, although inositol depletion is apparent during both acute and chronic treatment (8). Expression of INO1 decreased during acute treatment (30 min to 1 h) but increased in response to chronic VPA exposure.

We have previously shown that chronic exposure of yeast cells to VPA results in induction of the UPR by increasing the de novo synthesis of ceramide (9). In the current study, we examined the cellular response to acute VPA to gain insight into the mechanisms underlying perturbation of ceramide synthesis. A microarray analysis of gene expression in response to acute VPA treatment revealed dramatically increased expression of the long chain base (LCB) exporter RSB1. Increased intracellular levels of LCBs dihydrosphingosine (DHS) and phytosphingosine (PHS) are toxic to cells. Induction of Rsb1 leads to increased export of LCBs across

IMPase, inositol monophosphatase; SPT, serine palmitoyltransferase; DHS-1P, dihydrosphingosine-1-phosphate; PHS-1P, phytosphingosine-1-phosphate; LCBP, long chain base phosphate; qRT, quantitative RT.
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the plasma membrane, alleviating LCB toxicity (10). Up-regulation of RSB1 suggests that LCB levels are increased during acute VPA treatment.

In addition to exporting LCBs by Rsb1, cells maintain low levels of these intermediates by metabolizing them to other species that are exported across the plasma membrane. LCBs are phosphorylated to LCBPs (DHS-1P and PHS-1P) by sphingoid base kinases (11). Yor1 exports LCBPs across the plasma membrane (12). LCBPs are also cleaved by the lyase Dpl1 to generate fatty aldehyde and ethanolamine phosphate (13).

LCB synthesis is negatively regulated by Orm proteins, which physically interact with and inhibit the rate-limiting enzyme of ceramide synthesis, serine palmitoyltransferase (SPT) (14, 15). Phosphorylation of Orm proteins inhibits their association with SPT, resulting in increased de novo synthesis of LCBs (16, 17). In addition to negative regulation by Orm proteins, LCB levels are also controlled by Fen1 and Sur4, fatty acid elongases that catalyze the synthesis of very long chain fatty acids (C24–C26), which, along with PHS, are substrates for the synthesis of ceramide (18).

In the current study, we show for the first time that acute VPA-mediated inositol depletion increases the de novo synthesis of PHS by down-regulating Orm and fatty acid elongase levels. In response, cells up-regulate genes that export (RSB1, YOR1) and metabolize (DPL1) PHS, outcomes that are partially remediated by inositol. These findings have implications for the inositol depletion hypothesis of the therapeutic mechanism of action of VPA.

Results

VPA Increases the Expression of RSB1—To identify pathways that are affected by acute VPA treatment, we carried out a genome wide microarray study of wild type cells treated with 0.6 mM VPA for 30 min in the presence (I+) or absence (I−) of inositol. 592 genes exhibited more than 2-fold change in gene expression in I− and 542 genes in I+ in response to VPA, as summarized in supplemental Table S1. The most striking finding was dramatically increased expression of RSB1 in response to VPA. Subsequent quantitative real-time (qRT)–PCR analysis indicated that VPA increased mRNA levels of RSB1 47-fold in I− and 13-fold in I+ medium (Fig. 1A). The increase in RSB1 mRNA resulted in increased Rsb1 protein, as determined by Western blot analysis of Rsb1 levels in wild type cells expressing an HA-tagged RSB1 gene (Fig. 1B). As previously reported, a 43-kDa Rsb1 protein and 64–80-kDa glycosylated Rsb1 species were detected (14, 19). VPA treatment increased levels of both the glycosylated and non-glycosylated species of Rsb1. Both RSB1 mRNA and Rsb1 protein levels were increased to a greater extent in I− than in I+, suggesting that the induction was, at least in part, a response to inositol limitation. Increased expression of the exporter Rsb1 in the presence of VPA suggests that VPA increases LCB levels.

VPA Increases PHS Levels—Cells containing elevated levels of LCBs are expected to exhibit increased sensitivity to exogenous PHS. VPA did not increase sensitivity of wild type cells to PHS, presumably because elevated Rsb1 levels can effectively remediate an increase in the LCBs. However, rsb1Δ cells exhibited sensitivity to PHS in the presence of VPA, which was rescued by overexpression of RSB1 (Fig. 2). These findings suggested that PHS levels were increased in VPA-treated cells. Consistent with this, mass spectrometric analysis indicated that VPA treatment led to a 30% increase in PHS levels in wild type cells, and a 50% increase in rsb1Δ cells (Fig. 3). PHS1-P was also significantly elevated in rsb1Δ cells.

VPA Increases the Expression of Genes That Remediate Increased PHS Levels—In addition to Rsb1-mediated export of PHS across the plasma membrane, PHS toxicity is remediated by Yor1 export or Dpl1 degradation of LCBPs. VPA increased expression of YOR1 and DPL1 by 11- and 6-fold, respectively, in I− medium, and 6- and 3-fold, respectively, in I+ medium (Fig. 4). Taken together, these findings indicate that VPA increases PHS levels, which may account for the increased expression of YOR1, DPL1, and RSB1 to maintain subtoxic levels of PHS.

VPA Decreases Expression of ORM Negative Regulators and FEN1 and SUR4 Fatty Acid Elongase Genes—At least two mechanisms could explain increased PHS levels in VPA-treated cells. As discussed above, Orm proteins negatively regulate de novo synthesis of LCBs by inhibiting activity of the rate-limiting enzyme, SPT. Deletion of ORM genes leads to increased levels of PHS (14). In addition, fen1Δ and sur4Δ mutants, which are
defective in the synthesis of very long chain fatty acid substrates for the synthesis of ceramide, accumulate PHS (18). We observed that VPA decreased the expression of ORM1 and ORM2 in I⁻ medium and, to a lesser extent, in I⁺ medium (Fig. 5). Orm2 protein levels were also decreased in I⁻ (Fig. 5), but not in I⁺ (data not shown). Orm1 protein levels were not decreased (data not shown). Furthermore, VPA down-regulated the expression of FEN1 and SUR4 in I⁻ and, to a lesser extent, in I⁺ (Fig. 6). These findings were in agreement with a previous report that fen1Δ and sur4Δ mutants exhibit VPA sensitivity (9). These results suggested that the mechanism whereby VPA increases PHS levels is by decreasing both Orm2-mediated negative regulation of PHS synthesis as well as the synthesis of fatty acid substrates that, along with PHS, are converted to ceramide.

Inositol Depletion Increases PHS—VPA-mediated perturbation of expression of genes that remediate elevated PHS levels was rescued to some extent by supplementation with exogenous inositol, suggesting that VPA exerts effects on PHS synthesis via inositol depletion. To address this possibility, we examined the effect of inositol starvation on PHS synthesis in ino1Δ cells, which lack myo-inositol-3-phosphate synthase and cannot synthesize inositol. To do so, ino1Δ cells were cultured in I⁻ medium, transferred to I⁺ for 30 min, and LCB levels were quantified. Acute inositol starvation of ino1Δ cells resulted in a striking 2-fold increase in PHS levels (Fig. 7). Consistent with elevated PHS, inositol starvation of ino1Δ cells resulted in decreased expression of negative regulators ORM1 and ORM2 and fatty acid elongase genes FEN1 and SUR4, and increased expression of exporters RSB1 and YOR1 (Fig. 8). Taken together, these findings indicate that the VPA-mediated increase in PHS levels can be explained by inositol depletion.
In this report, we show that acute exposure to VPA increases de novo synthesis of PHS by inositol depletion. Our findings support a model (Fig. 9) whereby VPA-mediated inositol depletion down-regulates expression of Orm and fatty acid elongase genes, resulting in accumulation of PHS. In response, cells up-regulate genes that export (RSB1, YOR1) and metabolize (DPL1) PHS. These outcomes are partially remediated by inositol.

Our findings are consistent with studies showing that cells lacking ORM1 and ORM2 exhibit increased levels of PHS and Rsb1 (14). The ORM2 promoter contains the UASino activation sequence, which is controlled by inositol (14). Although chronic VPA-mediated inositol depletion increases expression of genes containing UASino in their
promoters, we have shown previously that acute exposure to VPA decreases expression of UAS\textsubscript{INO}\textsuperscript{-}containing genes, including \textit{INO1} and \textit{INO2} (8). Consistent with this, the current study shows that acute VPA treatment down-regulates the expression of \textit{ORM} genes, similar to other UAS\textsubscript{INO}\textsuperscript{-}containing genes.

\textit{fen1Δ} and \textit{sur4Δ} mutants have been shown to accumulate PHS (18). In agreement with these studies, decreased expression of \textit{FEN1} and \textit{SUR4} in VPA-treated cells is consistent with increased PHS levels.

The inositol-depleting drug lithium, which is also widely used to treat BD, does not decrease \textit{INO1} expression during acute treatment (8). This suggests that the decrease in expression of UAS\textsubscript{INO}\textsuperscript{-}containing genes during acute VPA treatment may be mediated via a mechanism other than inositol depletion. Ju and Greenberg (8) showed that acute VPA causes a decrease in the level of phosphatidylserine, a protein kinase C activator. Decreased activity of PKC causes an increase in unphosphorylated Opi1 (active), which leads to a decrease in \textit{INO1} expression (20). Consistent with this, acute VPA does not affect expression of UAS\textsubscript{INO}\textsuperscript{-}containing genes in \textit{opi1Δ} mutant cells. This model is in agreement with our observations and suggests that acute VPA may down-regulate expression of Orm2, a UAS\textsubscript{INO}\textsuperscript{-}containing gene, via Opi1.

The decrease in expression of \textit{ORM}, \textit{FEN1}, and \textit{SUR4} genes, and increase in expression of \textit{RSB1}, \textit{YOR1}, and \textit{DPL1} are greater in I\textsubscript{+}/H11002 than in I\textsubscript{+}/H11001 medium, suggesting that the response to VPA is partially mediated by inositol depletion. In agreement with this, acute inositol starvation of \textit{ino1Δ}/H9004 cells caused similar effects, including decreased expression of \textit{ORM}, \textit{FEN1}, and \textit{SUR4} genes, increased expression of \textit{RSB1} and \textit{YOR1}, and increased levels of PHS. Based on these data, we propose that perturbation of expression of these genes, leading to increased levels of PHS, is mediated by inositol depletion.

We have shown previously that chronic VPA treatment activates the UPR as a result of increased synthesis of ceramide containing PHS and C24–C26 fatty acids (9). The current study suggests that the mechanism underlying this outcome is down-regulation of Orm negative regulators and fatty acid elongases in the acute response to VPA, resulting in accumulation of PHS. \textit{FEN1} and \textit{SUR4} expression are increased during chronic exposure, resulting ultimately in increased synthesis of C24–C26 ceramide. Both acute and chronic effects are responses to inositol depletion.
Valproate Increases de Novo Synthesis of Phytosphingosine

TABLE 1

The mid-log phase (twice with sterile water, and grown in I)
thetic minimal medium with inositol (I)
growth in liquid cultures. All incubations were at 30 °C.
was added. Absorbance was measured at 550 nm to monitor

Experimental Procedures

Valproate Increases de Novo Synthesis of Phytosphingosine

This study shows for the first time that acute VPA-mediated inositol starvation increases intracellular levels of the signaling sphingolipid molecule, PHS. In yeast, PHS regulates actin cytoskeleton, heat stress, and endocytosis (21). Sphingosine, the mammalian LCB, activates synaptobrevin and membrane fusion. In addition, sphingosine plays a crucial role in increasing synaptic vesicle exocytosis at nerve terminals and neuromuscular junctions (22). We speculate that perturbation of sphingosine by VPA might affect neurotransmitter release and other processes that affect neural function and may, thus, be a novel mechanism of action of the drug.

Yeast Strains, Growth Medium, and Conditions—Strains used in this study are summarized in Table 1. Cells were maintained on YPD medium (2% glucose, 1% yeast extract, 2% bactopeptone). Deletion mutants were maintained on medium supplemented with G418 (200 μg/ml). Synthetic minimal medium without inositol (I−) contained all the essential components of Difco yeast nitrogen base (minus inositol), 2% glucose, 0.2% ammonium sulfate, vitamins, the four amino acids histidine (20 mg/liter), methionine (20 mg/liter), leucine (60 mg/liter), and lysine (20 mg/liter), and the nucleobase uracil (40 mg/liter). Where indicated, inositol (I+) was added at a concentration of 75 μM. For selection of plasmids, uracil was omitted. Liquid and solid media were supplemented with 0.6 mm and 1 mM VPA, respectively, when indicated. For solid media, 2% agar was added. Absorbance was measured at 550 nm to monitor growth in liquid cultures. All incubations were at 30 °C.

VPA Treatment—Wild type cells were precultured in synthetic minimal medium with inositol (I+), harvested, washed twice with sterile water, and grown in I− until the cells reached the mid-log phase (A550 = 0.5). Cells were pelleted, washed twice with sterile water and inoculated in I+ or I− to a final A550 of 0.05 and cultured until the cells reached the mid-log phase (A550 = 0.5). Cells were then pelleted and suspended in fresh I− or I+ medium with or without 0.6 mM VPA and incubated for 30 min.

ino1Δ Starvation—ino1Δ cells were precultured in I+ medium, harvested, washed twice with sterile water, and grown in I+ until the cells reached the mid-log phase (A550 = 0.5). Cells were pelleted, washed twice with sterile water and cultured in fresh I− (inositol starvation) or I+ (control) medium for 30 min.

Microarray Analysis—Total RNA was isolated by hot phenol extraction (23) and purified using an RNaseasy kit from Qiagen. Quality of RNA was determined using Agilent 2100 Bioana-
lyzer. RNA was labeled using the Agilent Low Input Quick-Amp labeling kit (Agilent Technologies). Cy3-labeled cRNA was then hybridized to the 8 × 15K Agilent Yeast V2 Arrays (design ID 016322). Slides were scanned on an Agilent G2505B microarray scanner and the resulting image files were processed with Agilent Feature Extraction software (version 9.5.1). All procedures were carried out according to the manufacturer’s protocols. Subsequent analysis was performed using GeneSpring (v10.0) software. Microarray analysis was carried out at the Research Technology Support Facility in Michigan State University.

qRT-PCR Analysis—Total RNA was extracted using the hot phenol method (23) and purified using an RNaseasy mini plus kit (Qiagen, Valencia, CA). Complementary DNA (cDNA) was synthesized using a first strand cDNA synthesis kit from Roche Applied Science, as described in the manufacturer’s manuals. qRT-PCR was done in a 20-μl volume reaction using Brilliant III Ultra-Faster SYBR Green qPCR master mix (Agilent Technologies, Santa Clara, CA). Each reaction was done in triplicate. The primers used for the qRT-PCR reactions are listed in Table 2. RNA levels were normalized to ACT1 levels (internal control). Relative values of mRNA transcripts are shown as fold-change relative to that of the indicated controls. Primers were validated as suggested in the Methods and Applications Guide (Agilent Technologies). The efficiencies of all primers used in this study were between 85 and 105%. Optimal primer concentrations were determined, and primer specificity of a single product was monitored by a melt curve following the amplification reaction. PCRs were initiated at 95 °C for 10 min for denaturation followed by 40 cycles consisting of 30 s at 95 °C and 60 s at 55 °C.

Long Chain Base Measurement—Cells were grown and treated with VPA as described above for 30 min, pelleted, and stored at −80 °C. Extraction of lipids from yeast pellets and lipid quantification by LC/MS/MS were performed as previously described (24).

Western Blotting—Cells were broken in the presence of acid-washed glass beads in lysis buffer containing 50 mM Tris, 125 mM sodium chloride, 1% Nonidet P-40, 2 mM EDTA, and 1× protease inhibitor mixture (Roche Applied Science). Extracts were centrifuged twice for 5 min at 13,000 × g at 4 °C to remove cell debris and glass beads. Protein concentration was determined using the Bradford assay (Pierce Protein), with bovine serum albumin as the standard protein. Proteins were resolved on 10 or 12% SDS-PAGE as indicated and electrotransferred to a polyvinylidene difluoride membrane (Millipore). The membrane was incubated with primary antibodies (1:3,000 anti-HA and anti-tubulin) and...
TABLE 2
Real-time PCR primers used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACT1</td>
<td>Forward</td>
<td>ACCGTCGACGCTTCATGACCTGTT</td>
</tr>
<tr>
<td>ACT1</td>
<td>Reverse</td>
<td>ACGTGTAGTCAACACACCATGCGAA</td>
</tr>
<tr>
<td>FEN1</td>
<td>Forward</td>
<td>TGGGTTTCCAACACTGCAACTGTT</td>
</tr>
<tr>
<td>FEN1</td>
<td>Reverse</td>
<td>CAAATCCGTTGCGCAACACCGG</td>
</tr>
<tr>
<td>SIR4</td>
<td>Forward</td>
<td>CTGTAGTCTACTGCTTGCT</td>
</tr>
<tr>
<td>SIR4</td>
<td>Reverse</td>
<td>AGTAGAAGACCGTGCTACAGG</td>
</tr>
<tr>
<td>RSB1</td>
<td>Forward</td>
<td>TGGGCTCCTCAATATGCTATTTCTTT</td>
</tr>
<tr>
<td>RSB1</td>
<td>Reverse</td>
<td>ACAGATGCTGGCGTTGGTGTCGA</td>
</tr>
<tr>
<td>YOR1</td>
<td>Forward</td>
<td>TCTCCAAGGACTGTCATTCCCTCA</td>
</tr>
<tr>
<td>YOR1</td>
<td>Reverse</td>
<td>TGCAACACTGCGATGTCGCTTG</td>
</tr>
<tr>
<td>DPL1</td>
<td>Forward</td>
<td>TCTCCGTAGTTGTGCGCTACATT</td>
</tr>
<tr>
<td>DPL1</td>
<td>Reverse</td>
<td>TGGTCCGACGACATTGTTGTCG</td>
</tr>
<tr>
<td>ORM1</td>
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<td>TGTTGCTACCAAGTGTCAGTT</td>
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<tr>
<td>ORM2</td>
<td>Forward</td>
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</tr>
<tr>
<td>ORM2</td>
<td>Reverse</td>
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</tr>
</tbody>
</table>

HRP-conjugated 1:10,000 anti-rabbit and anti-mouse secondary antibodies and visualized using ECL Plus substrate (Pierce Protein), with tubulin as the loading control.

Author Contributions—S. J. and M. L. G. designed the research and wrote the manuscript; S. J. carried out all the experiments; S. R. and A. C. performed long chain base measurements; S. J., A. C., and M. L. G. carried out the data analysis. All authors reviewed the results and approved the final version of the manuscript.

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Note Added in Proof—There were several errors in the version of this article that was published as a Paper in Press on January 18, 2017. An image was inadvertently duplicated in Fig. 2, and the wrong tubulin immunoblot was used to assemble Fig. 5B. These errors have now been corrected and do not affect the results or conclusions of this work.

References