Valproate Induces the Unfolded Protein Response by Increasing Ceramide Levels*§

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Bipolar disorder (BD), which is characterized by depression and mania, affects 1–2% of the world population. Current treatments are effective in only 40–60% of cases and cause severe side effects. Valproate (VPA) is one of the most widely used drugs for the treatment of BD, but the therapeutic mechanism of action of this drug is not understood. This knowledge gap has hampered the development of effective treatments. To identify candidate pathways affected by VPA, we performed a genome-wide expression analysis in yeast cells grown in the presence or absence of the drug. VPA caused up-regulation of FEN1 and SUR4, encoding fatty acid elongases that catalyze the synthesis of very long chain fatty acids (C24 to C26) required for ceramide synthesis. Interestingly, fen1Δ and sur4Δ mutants exhibited VPA sensitivity. In agreement with increased fatty acid elongase gene expression, VPA increased levels of phytoceramide, especially those containing C24–C26 fatty acids. Consistent with an increase in ceramide, VPA decreased the expression of amino acid transporters, increased the expression of ER chaperones, and activated the unfolded protein response element (UPRE), suggesting that VPA induces the UPR pathway. These effects were rescued by supplementation of inositol and similarly observed in inositol-starved ino1Δ cells. Starvation of ino1Δ cells increased expression of FEN1 and SUR4, increased ceramide levels, decreased expression of nutrient transporters, and induced the UPR. These findings suggest that VPA-mediated inositol depletion induces the UPR by increasing the de novo synthesis of ceramide.

Bipolar disorder (BD), one of the most severe forms of mood disorder, is characterized by recurrent episodes of depression and mania (1, 2). BD is ranked as the sixth leading cause of disability worldwide. It affects about 1–2% of the total world population (1–3) and leads to suicide in 15% of cases (4). Valproic acid (VPA), a branched short-chain fatty acid, is one of the most widely used drugs for the treatment of BD. However, it is effective in only 40–60% of cases and results in serious side effects, including hepatotoxicity and teratogenicity (5). Although many hypotheses have been postulated to explain its efficacy, the therapeutic mechanism of the drug is not understood, nor is the underlying cause of the disease (6, 7). This knowledge gap hampers the development of more effective drugs to treat BD.

The inositol depletion hypothesis has had a major impact on research in BD. Berridge (8) proposed that lithium, widely used to treat BD, inhibits inositol monophosphatase, causing inositol depletion and subsequently decreasing inositol 1,4,5-triphosphate-mediated signaling (8, 9). We have shown previously that VPA, similar to lithium, causes a decrease in intracellular inositol in yeast and mammalian cells (10–12). VPA indirectly inhibits myo-inositol-3-phosphate synthase (MIPS), the enzyme responsible for the rate-limiting step of de novo synthesis of inositol, suggesting that MIPS is post-translationally regulated (13). More recent findings show that yeast and human MIPS are phosphorylated (14, 15) and that phosphorylation of conserved sites affects enzymatic activity (14). These findings suggest that the mechanism by which VPA causes inositol depletion is conserved in yeast and mammals, supporting the yeast model for genetic and molecular studies of the mechanism of the drug.

In yeast, supplementation of inositol triggers a change in the expression of hundreds of inositol-regulated genes, including genes for lipid synthesis (16–18). Inositol-containing lipids, including inositol phosphoinositides, glycosylphosphatidylinositol, and sphingolipids, play crucial structural and functional roles in regulating membrane biogenesis, membrane trafficking, cytoskeletal organization, and gene expression (19–23). Hence, inositol depletion exerts profound effects on cellular function (24). Inositol depletion not only alters lipid biosynthesis (25) but also activates stress response pathways, including the protein kinase C and unfolded protein response (UPR) pathways (26–28). Cells grown in the absence of inositol exhibit induction of the UPR pathway (27, 29), which is reversed by inositol supplementation (16). These studies suggest that decreasing the intracellular levels of inositol induces the UPR pathway by a mechanism not yet characterized.
Valproate Induces the Unfolded Protein Response via Ceramide

Interdependence of the UPR pathway and ceramide synthesis has been demonstrated in yeast and mammals. Induction of the UPR increases ceramide levels and viability in yeast (30). In mammals, activation of the UPR increases the expression of ceramide synthase CerS6, leading to increased synthesis of ceramides containing C16 fatty acids (30). Perturbation of de novo synthesis of sphingolipids activates the UPR in yeast (29, 31), and defective ceramide homeostasis leads to UPR failure (32). Ceramide also decreases the transcription of nutrient transporters, including amino acid transporter mCAT-1, glucose transporter GLUT-1 (33), glucose transporter HXT4, and uracil permease FUR4 (34). Therefore, the interrelationship between ceramide levels and the UPR pathway maintains cell homeostasis.

In the current study, we show for the first time that VPA-mediated inositol depletion induces the UPR pathway by increasing de novo synthesis of ceramide, especially C24–C26-containing phytoceramide. These findings have implications for the therapeutic mechanism of VPA.

**Results**

**VPA Increases the Expression of Fatty Acid Elongases**—To determine candidate pathways that may be important for the therapeutic role of VPA, we performed a genome-wide microarray analysis of cells treated with 0.6 mM VPA for 5 h in the presence or absence of inositol, as described under “Experimental Procedures.” VPA treatment resulted in altered expression (>2-fold) of 324 genes in the presence of inositol and 413 genes in the absence of inositol (supplemental Table 1). Interestingly, fatty acid elongase genes FEN1 and SUR4 exhibited 2-fold increased expression in response to VPA (supplemental Table 1). qRT-PCR analysis of fatty acid elongase genes in WT cells treated with VPA validated these findings. As seen in Fig. 1, mRNA levels of FEN1 and SUR4 were increased 6- and 4-fold in the absence of inositol and to a lesser extent (3- and 2-fold) in the presence of inositol. Fen1 and Sur4 catalyze the synthesis of very long chain fatty acids, including C22 and C24 (Fen1) and C24 and C26 (Sur4) (35), which are used for the synthesis of ceramide. However, VPA did not alter the expression levels of ELO1, a fatty acid elongase that catalyzes the synthesis of C16 or C18 (Fig. 1). This indicates that VPA specifically increases the expression of fatty acid elongases catalyzing the synthesis of very long chain fatty acids (C24–C26). Mutants fen1Δ and sur4Δ exhibited sensitivity to VPA, as did the sphingamine C4-hydroxylase mutant, sur2Δ (Fig. 2). VPA sensitivity was partially rescued by inositol. The VPA-mediated increase in the expression of these genes and the VPA sensitivity of Fen1, Sur4, and Sur2 mutants suggested that VPA induces an increase in ceramide containing C24–C26 fatty acids and phytosphingosine (the product of Sur2).

**VPA Increases Ceramide Levels and Down-regulates Amino Acid Transporters**—To determine whether VPA increases ceramide levels, dihydroceramide (DHC) and phytoceramide (PHC) ceramide species were analyzed by mass spectrometry in WT cells. VPA showed a trend of increase in ceramide levels, dihydroceramide (DHC) and phytoceramide (especially C26 phytoceramide, but this increase was not statistically significant (Fig. 3B). Interestingly, VPA treatment for 30 min up-regulated the expression of RSB1 (data not shown), which transports long chain bases (LCBs) (including dihydrospingosine and phytosphingosine) across the plasma membrane from the inside to the outside of the cell (36). In WT cells, up-regulation of RSB1 increases the transport of LCBs, reducing the intracellular levels of these ceramide precursors. Therefore, we measured the effect of VPA on ceramide levels with varying fatty acids in rsb1Δ cells. VPA increased both DHC and PHC in rsb1Δ (Fig. 3). Specific ceramide species were increased, including DHC with C16, C18,
and C20 and PHC with C16, C24, and C26 in rsb1Δ cells (Fig. 3). The highest increase was observed in C26 species of PHC. The likely explanation for this is that the increase in RSB1 in WT cells reduces the available LCB precursor for ceramide synthesis. However, when RSB1 is deleted, the increase in ceramide levels is more significant.

Previous studies have shown that increased levels of ceramide cause a decrease in the transcription of nutrient permeases, leading to reduced intake of nutrients and induction of stress (33, 34). Consistent with this, the microarray analysis revealed decreased expression of amino acid transporter genes BIO5, AGP1, GAP1, BAP2, DIP5, UGA4, CAN1, and SAM3 in response to VPA in the absence of inositol. The expression of these transporters was not altered in I+/H11001 medium (supplemental Table 1). Decreased expression of amino acid transporters was confirmed by qRT-pCR in both WT and rsb1Δ cells (Fig. 4). Down-regulation was greater in rsb1Δ than in WT cells, consistent with the increase in ceramide levels. To determine whether decreased expression of amino acid transporters resulted from increased ceramide, cells were treated with 100 μM fumonisin, a ceramide synthase inhibitor (37, 38). Fumonisin reversed the down-regulation of the amino acid transporters in both WT and rsb1Δ cells (Fig. 4). Taken together, these findings suggest that VPA increases levels of specific ceramide species, resulting in decreased expression of nutrient permeases.

**VPA Induces the UPR by Increasing Ceramide Levels—**
Decreased expression of nutrient transporters is expected to induce stress due to nutrient starvation (33). In agreement with this, VPA increased the expression of ER chaperone genes ELI1, JEM1, KAR2, LHS1, SEC63, and PDI1 in I− medium (supplemental Table 1 and Fig. 5), suggesting that the UPR pathway was induced. To test this, we analyzed the UPR response in WT and rsb1Δ cells expressing a UPR-lacZ reporter plasmid. Increased lacZ activity was observed in response to VPA in I− medium (Fig. 6B). The increase was more pronounced in rsb1Δ than in WT cells. Fumonisin, a
Valproate Induces the Unfolded Protein Response via Ceramide

In this study, we show that VPA induces the UPR by increasing ceramide levels via inositol depletion. Our specific findings indicated that VPA 1) increased expression of fatty acid elongases synthesizing C24 and C26 fatty acids; 2) increased ceramide levels, particularly PHC with C24–C26 fatty acids; 3) decreased the expression of amino acid transporters; and 4) induced the UPR pathway. These outcomes were abrogated by inhibition of de novo ceramide synthesis or by supplementation of inositol.

Ceramide levels can be increased via two routes, increased de novo synthesis by ceramide synthase or inhibition of inositol phosphorylceramide synthase, which converts ceramide to complex sphingolipids. Our findings suggest that VPA induces the UPR pathway by increasing de novo synthesis of ceramide (Fig. 6B) as a result of increased expression of fatty acid elongases, because induction did not occur in the presence of the ceramide synthase inhibitor fumonisin (37). In contrast, aureobasidin, which inhibits inositol phosphorylceramide synthase, did not affect VPA-mediated induction of the UPR (Fig. 6C).

Our findings are consistent with studies showing that inositol starvation induces the UPR. UPR target genes were found to be significantly up-regulated in cells grown in the absence of inositol (16). In addition, inositol supplementation alters the expression of UPR pathway genes (39). Although initial studies showed that accumulation of unfolded proteins in the ER induces the UPR pathway (40, 41), it has since been determined that Ire1p, the transmembrane kinase that senses ER stress (27, 42), induces the UPR pathway in response to changes in membrane lipid composition (29). In this light, inositol starvation may trigger the UPR as a result of lipid-related membrane changes in the ER, including perturbation of sphingolipid metabolism. The addition of inositol induces changes in the synthesis and levels of numerous lipids, including sphingolipids (28, 43). Recent studies show that perturbation of sphingolipid metabolism may affect ER membrane homeostasis. Mutant cells lacking ORM1 and ORM2, negative regulators of sphingolipid metabolism (31, 44), exhibit constitutive induction of the UPR response (31). In addition, orm1Δ orm2Δ cells contain elevated levels of sphingolipids (31, 44) and are hypersensitive to stress induced by inositol starvation (31). Similarly, UPR expression is constitutive in isc1Δ mutant cells, which contain elevated sphingolipids due to a block in sphingolipid turnover (45, 46). Conversely, inhibiting sphingolipid synthesis with myriocin treatment suppresses activation of the UPR induced by inositol starvation (29). Elevated sphingolipids in the ER may lead to membrane aberrancy that activates the UPR pathway independent of accumulation of unfolded protein. The role of specific sphingolipids in activation of the UPR is not known.
Our study suggests that accumulation of ceramide, specifically C24–C26-containing PHC, induces the UPR upon inositol depletion.

We have previously shown that VPA decreases intracellular inositol levels (10–13, 47). VPA induction of the UPR occurred only in I−/H11002 conditions, suggesting that the response to VPA was mediated by inositol depletion. Consistent with this, inositol starvation of ino1/H9004 cells caused similar effects, including increased expression of fatty acid elongases, increased intracellular levels of ceramides, especially PHC with C24 and C26, down-regulation of nutrient transporters, and induction of the UPR. Based on these data, we propose the following model (Fig. 8). VPA decreases intracellular inositol levels, causing an increase in C24 and C26 PHC species as a result of up-regulation of expression of fatty acid elongases (FEN1 and SUR4). This results in increased ceramide synthesis, which causes down-regulation of expression of nutrient transporters, inducing stress and the UPR.

Several studies suggest that the UPR pathway may be involved in the pathophysiology of BD. Lymphoblasts from bipolar patients show an aberrant ER stress response (48, 49). Compared with cells from healthy controls, lymphoblasts from bipolar patients had lower expression of XBPI (mammalian homologue of yeast HAC1), the spliced form of which binds the UPRE element and induces the UPR pathway in response to ER stress inducers (49). A polymorphism (−116C → G) in the XBPI promoter that is associated with lower gene transcription was observed in BD lymphoblastoid cells (50). These findings suggest that the UPR pathway may be perturbed in bipolar patients, and activation of this pathway may be important for the therapeutic action of VPA. In this light, the UPR pathway may be a possible new target for BD therapy.

**Experimental Procedures**

**Yeast Strains, Growth Media, 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 of the essential components of Difco yeast nitrogen base (minus...
Valproate induces the Unfolded Protein Response via Ceramide

in insulin); 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 and 1 mM VPA, respectively, when indicated. Fumonisin B1 (Sigma) and aureobasidin A (Clontech) were used at a concentration of 100 μM and 0.5 g/l, respectively. 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—WT cells were precultured in synthetic minimal medium with inositol (I10000), harvested, washed twice with sterile water, and grown in I10001 until the cells reached the mid-log phase (A550 = 0.5). Cells were then pelleted and suspended in fresh I10002 or I10001 medium with or without 0.6 mM VPA and incubated for 5 h.

FIGURE 7. VPA induces the UPR by inositol depletion. ino1Δ cells expressing the UPRE-lacZ reporter plasmid were precultured and grown in Ura-I+ medium until the cells reached mid-log phase (A550 = 0.5). Cells were washed and transferred to Ura-I− medium with or without 100 μM fumonisin for 3 h. A, LacZ activity. Data shown are mean ± S.D. (error bars) (n = 6) (*, p < 0.05; **, p < 0.01; ***, p < 0.001). B, DHC and PHC levels with varying fatty acids were quantified by mass spectrometry. Data shown are mean ± S.D. (n = 6) (*, p < 0.05; **, p < 0.01; ***, p < 0.001). mRNA levels of nutrient transporters (C) and fatty acid elongases (D) were quantified by qRT-PCR, as indicated. Values are reported as -fold change in expression in cells grown in I− medium relative to cells grown in I+ medium. Expression was normalized to the mRNA levels of the internal control ACT1. Data shown are mean ± S.D. (n = 6). *, p < 0.05; **, p < 0.01; ***, p < 0.001.

VPA Treatment—WT 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+ 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 5 h.

FIGURE 8. Model; VPA induces the UPR pathway by increasing intracellular ceramide levels. In the proposed model, VPA-mediated inositol depletion leads to increased expression of fatty acid elongases, specifically FEN1 and SUR4, which catalyze the synthesis of C24 and C26 fatty acids and ceramides, especially PHC containing C24–C26 fatty acids. Increased ceramide levels decrease expression of nutrient transporters, stressing the cell due to lack of nutrients and inducing the UPR pathway.
Valproate Induces the Unfolded Protein Response via Ceramide

TABLE 1
Yeast strains and plasmids used in this study

| Strain/Plasmid          | Genotype/Description | Source/Reference |
|-------------------------|----------------------|------------------|
| Wild type               | MATa, his 3Δ, ura 3Δ | Invitrogen       |
| fen1Δ                   | MATa, his 3Δ, ura 3Δ, fen1Δ: KanMX4 | Invitrogen       |
| sur1Δ                   | MATa, his 3Δ, ura 3Δ, sur1Δ: KanMX4 | Invitrogen       |
| rsi1Δ                   | MATa, his 3Δ, ura 3Δ, rsi1Δ: KanMX4 | Invitrogen       |
| ino1Δ                   | MATa, his 3Δ, ura 3Δ, ino1Δ: KanMX4 | Invitrogen       |
| RSB1-HA                 | pRS316-BSB1Δ353-382-HA | Johnson et al. (55) |
| UPRE-LacZ               | pC194, containing UPRE-CYC-lacZ | Chang et al. (53) |

TABLE 2
Real-time PCR primers used in this study

| Gene          | Primer                  | Sequence                  | (5’–3’)                  |
|---------------|-------------------------|---------------------------|--------------------------|
| ACT1          | Forward                 | AGCTTCCAGCCTTCTAGTCTTCA   |                          |
| ACT1          | Reverse                 | ACGTTGAAGAACGACTGCACGAA   |                          |
| FEN1          | Forward                 | TGGGTTCACCAACCTGACCTTCT   |                          |
| FEN1          | Reverse                 | TCATTACCTCTCGCGGAACTGAT   |                          |
| SUR4          | Forward                 | TGTATGCTGACCTGCTGCTGCT    |                          |
| SUR4          | Reverse                 | AGTAGAGAAACGAGGATGCAAGGAG|                          |
| RSBI          | Forward                 | TTGGCCTCTCCAAATGGCTATCT   |                          |
| RSBI          | Reverse                 | ACGTGTTCCGGTGTTGCTGAC     |                          |
| ELO1          | Forward                 | GAAGAACCTTCTAGTGGTCACCAA  |                          |
| ELO1          | Reverse                 | GAGATCCTCTACGGCTGCTATC    |                          |
| GAP1          | Forward                 | GTGACATCTCAGGGCTTACAA     |                          |
| GAP1          | Reverse                 | GACAGCAAGGACCAACATTCTC   |                          |
| BAP2          | Forward                 | GGAAGCTGGGCTGAGTCTATC     |                          |
| BAP2          | Reverse                 | GCCCAATACCTGCTGCCAAAG     |                          |
| DIP5          | Forward                 | TCAATTTGTCGACCTGTTACA     |                          |
| DIP5          | Reverse                 | GGTCCCTTCATCCCTCCCTCTCTCT|                          |
| UGA4          | Forward                 | AGCGAGGATGAGGAAGGCTTCT    |                          |
| UGA4          | Reverse                 | AGCGAGGATGAGGAAGGCTTCT    |                          |
| CAN1          | Forward                 | Gaagctttgagtaagagagagagag|                          |
| CAN1          | Reverse                 | GATGATGACGCTTCTCTCTCTCTCT|                          |
| SIM3          | Forward                 | GATGGAATGACGCTTCTCTCTCTCT|                          |
| SIM3          | Reverse                 | GATGGAATGACGCTTCTCTCTCTCT|                          |
| EUG1          | Forward                 | TGTTCACTGCTTCGGGCTGTGCAA |                          |
| EUG1          | Reverse                 | CTTAAGACTCCGTGACGCAAAG    |                          |
| JEM1          | Forward                 | GCGTGTTGGCAGCTCCCAACAG    |                          |
| JEM1          | Reverse                 | GCATGGGCTGCAGCTGCAGAAG    |                          |
| KAR2          | Forward                 | AAGATGGGAGGACGCGTCGAGAAG  |                          |
| KAR2          | Reverse                 | ACAAGATGGAACGCTGAGCAGG    |                          |
| LHS1          | Forward                 | AGCCAGACCTGGAGAACGACTTAT |                          |
| LHS1          | Reverse                 | AGCCAGACCTGGAGAACGACTTAT |                          |
| SEC63         | Forward                 | AGCGAAGGCCTAACAATGGCAGAAG|                          |
| SEC63         | Reverse                 | TGGGCACTGGGATGAGCAGGATTT |                          |
| PDH1          | Forward                 | TGCCATCAGCAGGCTCGAGAAG    |                          |
| PDH1          | Reverse                 | ACGTGTTCCGGTGTTGCTGAC     |                          |

ino1Δ Starvation—ino1Δ cells were precultured in I−, harvested, washed twice with sterile water, and grown in I+ until the cells reached mid-log phase (A550 = 0.5). Cells were pelleted, washed twice with sterile water, and transferred to fresh I− (inositol starvation) or I+ (control) for 3 h.

Microarray Analysis—Total RNA was isolated by hot phenol extraction (51) and purified using an RNaseasy kit from Qiagen. Quality of RNA was determined using Agilent 2100 Bioanalyzer. RNA was labeled using the Agilent Low Input QuickAmp 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 (version 10.0) software. Microarray analysis was carried out at the Research Technology Support Facility at Michigan State University.

qRT-PCR Analysis—Total RNA was extracted using the hot phenol method (51) and purified using an RNaseasy mini plus kit (Qiagen, Valencia, CA). cDNA was synthesized using the first strand cDNA synthesis kit from Roche Applied Science as described in the manufacturer’s manuals. qRT-PCRs were done in a 20-μl volume reaction using Brilliant III Ultra-Fast SYBR Green qPCR master mix (Agilent Technologies, Santa Clara, CA). Each reaction was done in triplicate. The primers used for the qRT-PCRs 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 the indicated controls. Primers were validated as suggested in the Methods and Applications Guide (Agilent Technologies). All primers used in this study had primer efficiency 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.

Ceramide Measurement—Cells were grown and treated with VPA as described above for 5 h, pelleted, and stored at −80 °C. Extraction of lipids from yeast pellets and lipid quantification by LC/MS/MS was performed as described previously (52).

β-Galactosidase Assays—Cells expressing the UPRE-lacZ reporter plasmid provided by Dr. Susan Henry (53) were precultured in Ura− and grown in Ura+ to an A550 of 0.5, washed, and transferred to Ura− medium with or without VPA for 5 h at 30 °C. Cells were harvested, and β-galactosidase was assayed as described (54).

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

Acknowledgments—We thank Dr. Susan Henry for the UPRE-lacZ reporter plasmid, Dr. Jeff Landgraf and the Research Technology Support Facility at Michigan State University for assistance in carrying out the microarray analysis, and Dr. Yusuf Hannun for valuable insights.

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Valproate Induces the Unfolded Protein Response via Ceramide

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and Miriam L. Greenberg

J. Biol. Chem. 2016, 291:22253-22261.
doi: 10.1074/jbc.M116.752634 originally published online September 1, 2016

Access the most updated version of this article at doi: 10.1074/jbc.M116.752634

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