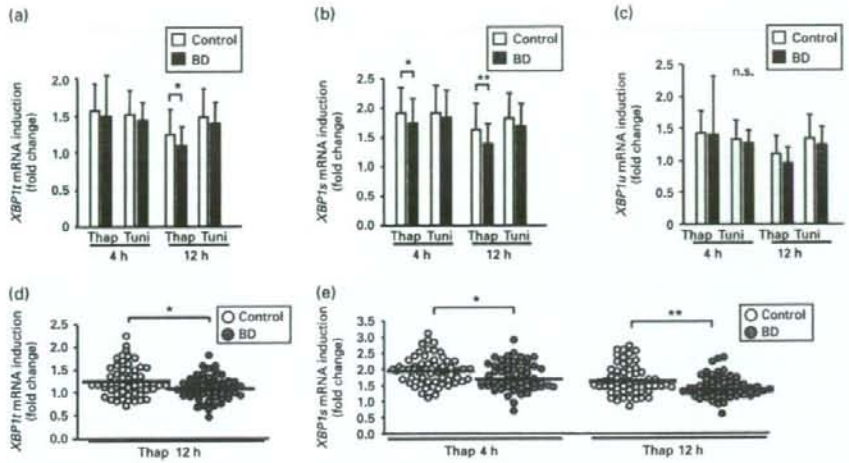

Figure 1. Altered endoplasmic reticulum (ER) stress-induced expression of *XBP1* mRNA in patients with bipolar disorder (BD). Bars represent means ± S.D. n.s., Not significant; * $p<0.05$, ** $p<0.01$ by Mann–Whitney *U* test. *XBP1t*, *XBP1* total; *XBP1s*, spliced form of *XBP1*; *XBP1u*, unspliced form of *XBP1*. Thap, thapsigargin; Tuni, tunicamycin. Bars in panels (d) and (e) represent the average.

Mann–Whitney *U* test). Similarly, the BD group exhibited significantly reduced induction of *XBP1s* at both 4 h and 12 h (Figure 1b, e; $p=0.027$, $p=0.008$ in Mann–Whitney *U* test, respectively). There was no significant difference in *XBP1u* induction between BD patients and controls (Figure 1c). Two-way ANCOVA showed a significant main effect of diagnosis, but not sex, for these parameters (*XBP1t* 12 h after thapsigargin, *XBP1s* 4 h after thapsigargin, and *XBP1s* 12 h after thapsigargin) and *XBP1u* 12 h after thapsigargin (Table S1, online).

There was no statistically significant difference of *XBP1* inductions between the genotypes of *XBP1* −116 C/G polymorphism (C/C, $n=12$; C/G, $n=45$; G/G, $n=61$, Kruskal–Wallis test, $p>0.05$). Three-way ANCOVA showed a significant interaction between diagnosis and genotype for *XBP1t* 12 h after tunicamycin. A trend of diagnosis × genotype interaction was found for *XBP1s* 12 h after tunicamycin, and *XBP1u* 4 h after tunicamycin (Table S3, online, Figure 2).

GRP94

GRP94 induction 12 h after thapsigargin treatment was also significantly reduced in BD patients (Figure 3a, b). Two-way ANCOVA also revealed a significant effect of diagnosis ($p=0.005$), but not sex, on GRP94 induction (Table S1, online).

We previously reported that the haplotype of *GRP94* was associated with BD, which was replicated in two independent Japanese sample sets (Kakiuchi et al., 2007). The protective haplotype (haplotype C-3) was determined by two SNPs (rs1165681 and rs17034977). The LB cells with haplotype C-3 exhibited significantly higher *GRP94* induction than those without this haplotype (Figure 3c; $p=0.013$ in Mann–Whitney *U* test). There was a trend of lower basal level of GRP94 in subjects with haplotype C-3 ($n=17$, 0.0186 ± 0.0072) compared to those without haplotype C-3 ($n=101$, 0.0226 ± 0.0097, $p=0.093$). The single SNP genotype of rs17034977 did not show significant effect on the basal GRP94 level and fold change of GRP94 response.

Three-way ANCOVA with factors of GRP94 haplotype, diagnosis, sex, and a covariate of age showed a trend level effect of GRP94 haplotype for basal level of GRP94 (Table S3, online), but there was not significant interaction between GRP94 haplotype and diagnosis for basal GRP94 levels and GRP94 induction.

Other UPR-related genes

There was no significant difference in the response of *GRP78*, *CALR*, and *CHOP* between BD patients and controls (Figure 4). *GRP78* has four haplotypes, among which haplotype 4 was reported to be associated with BD in the Japanese population. Although we compared the induction of *GRP78* with or without haplotype 4, no significant difference was observed (data not shown).

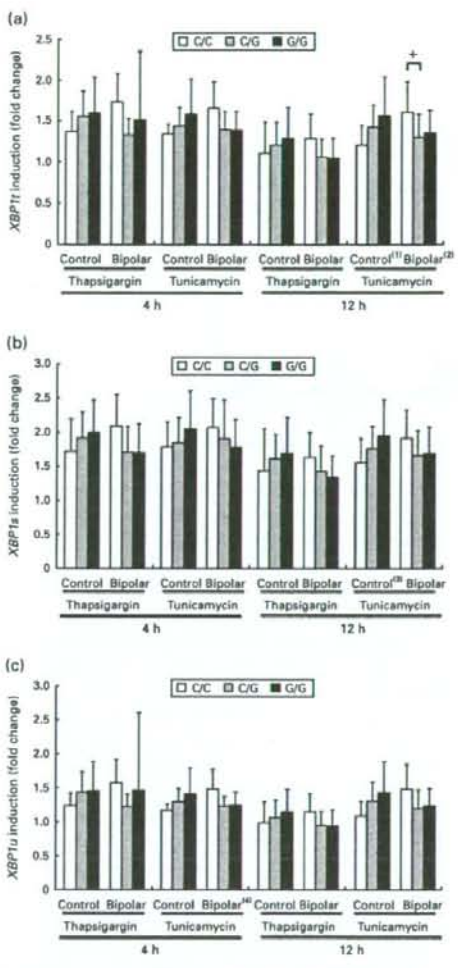

Figure 2. Effect of *XBP1* −116 polymorphism on XBP1 induction. Bars represent means ± S.D. *XBP1t*, *XBP1* total; *XBP1s*, spliced form of *XBP1*. Superior numbers indicate significance or a trend of genotype effect observed by one-way ANOVA. (1) $F=2.548$, $p=0.087$, (2) $F=2.543$, $p=0.088$, (3) $F=2.567$, $p=0.086$, (4) $F=4.307$, $p=0.018$. $^{+}$ $p<0.10$ by multiple comparison by Bonferroni method.

Effects of clinical parameters

Effect of clinical variables on the ER stress response was examined. There was no significant relationship between age at onset and any of the gene expression parameters in the BD group ($p>0.05$). None of the parameters was significantly different between the patients with and without a history of psychosis ($p>0.05$). With regard to family history, several parameters showed nominally significant difference between patients with and without a family history. Especially, response of CHOP to both thapsigargin and tunicamycin was significantly different between the two groups. Patients with a family history showed significantly smaller response to these two agents compared to those without a family history (Table S4, online).

Discussion

We found that induction of *XBP1t* and *XBP1s* by thapsigargin was significantly reduced in the BD group in accordance with previous findings (Kakiuchi et al., 2003; So et al., 2007) (Figure 1). We also found that induction of an ER chaperone gene, *GRP94* (*HSP90B1*), in response to thapsigargin was also attenuated in BD (Figure 3). There was a non-significant tendency of higher basal expression levels of *XBP1s*, *XBP1u*, and *XBP1t* in BD patients (Table 2). These expression levels were statistically significantly higher in BD I patients compared to controls. These findings imply that LB cells from BD, especially BD I, might mildly but constitutively suffer from ER dysfunction even in the basal condition and could not trigger enough appropriate *XBP1* response to maintain ER homeostasis on stress condition. The results should be interpreted with caution, because the patient group is heterogeneous, and LB cells are polyclonal. The major strength of this study, in contrast to previous studies, is the use of a relatively large number of LB cells and the carefully designed measurement method for the distinction between *XBP1s* and *XBP1u* mRNAs (Figure S1, online).

We previously reported that this polymorphism on the promoter region of *XBP1* removes the binding site of XBP1 itself and compromises the *XBP1* induction in response to ER stress (Kakiuchi et al., 2003). In our previous study, induction of *XBP1t* 3 h after the 300-nM thapsigargin stimulation was 2.6-fold in C/C genotype cells of control subjects. In the study by So et al. (2007) it was 5-fold in the control cells with C/C genotype 6 h after the 300-nM thapsigargin treatment. In contrast, the induction of *XBP1t* after the 4-h treatment with 300 nM thapsigargin in the present study was only 1.5-fold in the cells with the same genotype. This was similar to or even lower than that in the G/G cells in previous studies. Thus, the XBP1 loop showed a different functional status in the present experimental conditions in comparison with the previous studies. In this study, we used the cells after two rounds of freezing and reculturing. Our preliminary

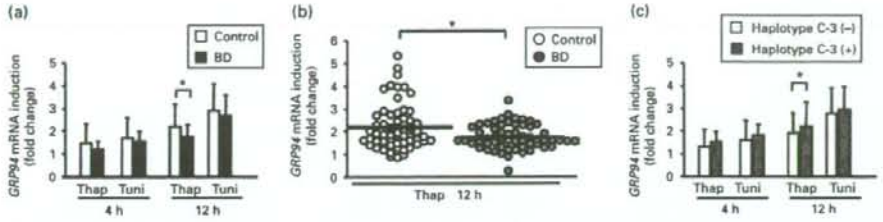

Figure 3. Induction of *GRP94* in lymphoblastoid cells. (a, b) Altered endoplasmic reticulum (ER) stress-induced expression of *GRP94* mRNA in patients with bipolar disorder (BD). (c) Difference of thapsigargin-induced *GRP94* response between the subjects with haplotype C-3 of *GRP94* ($n=17$) and those without haplotype C-3 ($n=101$). Bars represent means ± S.D. * $p<0.05$, by Mann–Whitney *U* test. Thap, thapsigargin; Tuni, tunicamycin.

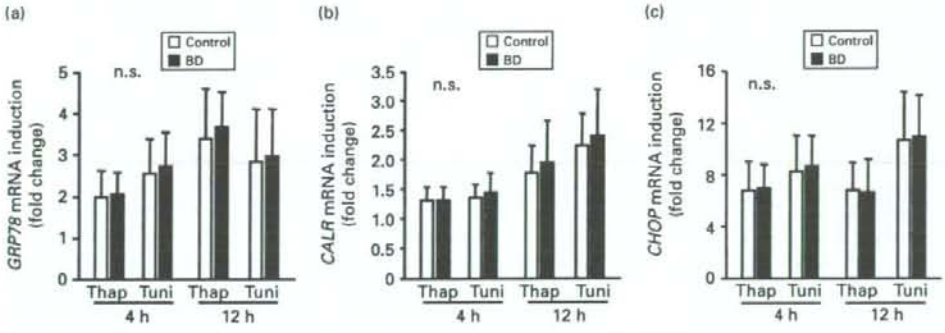

Figure 4. Induction of *GRP78*, *CALR*, and *CHOP* in lymphoblastoid cells. Bars represent means ± S.D. n.s., Not significant; BD, Bipolar disorder; Thap, thapsigargin; Tuni, tunicamycin.

experiment showed that *XBP1t* response to thapsigargin was attenuated after the freezing and reculturing process (Figure S2, online). Despite this limitation, we used the cells after two rounds of freezing and reculturing to increase the sample size, because only such cells were available for many of the previously collected cell lines. Although the molecular mechanism of alterations in the UPR in LB cells after freezing and reculturing is unknown, freezing of the cells may cause unfolding of the proteins resulting in ER stress. Caution should be exercised in directly comparing the results of the present study with those of previous studies.

It is of interest that we were able to observe the difference in *XBP1* response to thapsigargin between BD patients and controls even in those cells with compromised *XBP1* response. The present findings, together with the finding by So et al. (2007) indicate that the effect of BD on the response of *XBP1* to ER stress surpasses the effect of the *XBP1* polymorphism, and impaired *XBP1* response to ER stress in BD cannot be solely attributable to this promoter SNP.

An unexpected finding was the significant interaction between diagnosis and *XBP1* genotype for *XBP1* induction by tunicamycin. This interaction seems to be mediated by the opposite relationship of genotype and *XBP1* response between BD patients and controls (Figure 2a). The XBP1 promoter undergoes complex regulation. NF-Y is constitutively bound to the *XBP1* promoter (Donati et al., 2006), and interacts with ATF6 (Yoshida et al., 2000). Many other transcription factors were also found to bind to the XBP1 promoter by chromatin immunoprecipitation assay (Donati et al., 2006). XBP1 itself is one of the transcription factors that directly bind to XBP1 promoter (Acosta-Alvear et al., 2007; Donati et al., 2006). *XBP1* expression is also epigenetically regulated by histone acetylation (Donati et al., 2006). Donati and colleagues hypothesized that XBP1u protein, an inactive transcription factor, is constitutively bound to *XBP1* promoter, and this is replaced by XBP1s, an active transcription factor upon ER stress (Donati et al., 2006). The differential response to ER stress may be caused by the difference in transcription factors bound to the *XBP1* promoter. Further

studies will be needed to understand this complex relationship between genotype and diagnosis.

On the other hand, the protective haplotype of *GRP94* against BD (Kakiuchi et al., 2007) displayed significantly higher induction of *GRP94* in the presence of thapsigargin, suggesting that the increased mRNA expression by the associated haplotype could be an underlying protective mechanism for BD. The present findings suggest that the UPR dysfunction in BD is not limited to the impaired feedback loop of *XBP1* as suggested by our previous study but might reflect broader dysfunction of this pathway.

It should be noted that several BD patients were outliers ($p < 0.10$ by Smirnov–Grubbs test) with regard to the *XBP1* or *GRP94* induction (Figures 1 and 3). A recent whole genome association study did not find genes robustly associated with BD (Wellcome Trust Case Control Consortium, 2007). The effect of copy number variations (CNVs) on the inter-individual variations of gene expression levels cannot be overlooked (Stranger et al., 2007). In this situation, the role of multiple rare variants in the pathophysiology of BD is revisited (Kato, 2007). It is possible that not only the combination of common SNPs but also rare mutations or CNVs of the genes in the UPR pathway might contribute to the reduced ER stress response in BD. Although the large number of molecules participating in this pathway hampers the re-sequencing study, recent innovation in re-sequencing technology will enable a search for rare mutations in this pathway in the near future.

It is unknown why other transcription target genes of XBP1, such as *GRP78*, *CALR*, and *CHOP*, were not altered in LB cells from BD patients. Our finding is in contrast to the results of Kakiuchi et al. (2003), who showed reduced induction of *GRP78* in response to thapsigargin, and So et al. (2007), who reported reduced CHOP expression in response to thapsigargin and tunicamycin in LB cells derived from BD patients. These discrepancies are probably due to the difference of the culture conditions or patient population. Although we did not find a significant difference of CHOP, we found a significant effect of family history on the response of CHOP. CHOP response was significantly smaller in patients with a family history. This may be relevant to the data of So et al. The discrepancy in the GRP78 response between this study and Kakiuchi et al. (2003) may be due to the difference in the number of freeze and reculture, because GRP78 response was enhanced after this process (Figure S2, online).

Although these genes have a *cis*-acting ER stress response element (ERSE) in their promoter, to which XBP1 directly binds (Yamamoto et al., 2004), these genes are not regulated solely by XBP1. There might be complicated and parallel signalling pathways for the regulation of these genes.

BD should be caused primarily by brain dysfunction. Thus, it is unlikely that changes of *XBP1* and *GRP94 in* peripheral blood cells directly cause neurological dysfunction. However, if the observed change in LB cells is the intermediate phenotype associated with genetic predisposition to BD, it seems plausible that such changes also occur in neuronal cells. There are three possible explanations for how impaired ER stress response is relevant to impaired neural function in BD: (1) roles of XBP1 in neural development and plasticity, (2) roles of UPR in the maturation and trafficking of receptors, and (3) roles of ER chaperones in calcium signalling.

The first possibility was suggested by our recent finding that *XBP1* is dramatically spliced by application of brain-derived neurotrophic factor (BDNF) in mouse primary hippocampal neurons (Hayashi et al., 2007). We suggested that BDNF increases protein synthesis, which triggers the ER stress condition in neurites and induces *XBP1* splicing. BDNF-induced neurite extension and branching was impaired in neurons lacking in *XBP1*. Although it is an open question whether the neurons of BD patients have impaired *XBP1* induction in response to BDNF, the impaired UPR may be one of several factors that together disrupt neuroplastic responses in BD. Decreased levels of BDNF in the serum of BD patients also support this possibility (Cunha et al., 2006).

With regard to the second possibility, the roles of ER chaperones on the maturation of membrane proteins involved in neurotransmitter signalling have been recognized since the report on the role of GRP78 in the function of the serotonin transporter (Tate et al., 1999). More recently, the requirement of XBP1 for surface trafficking of GLR-1, a *C. elegans*, ionotropic glutamate receptor most similar to the mammalian AMPA receptor, has been reported (Shim et al., 2004).

ER stores Ca^{2+} that is used for Ca^{2+} signals (Meldolesi and Pozzan, 1998). Most of the ER chaperones such as GRP78 and GRP94 have Ca^{2+}-binding capacity, which regulates Ca^{2+} flux (Yu et al., 1999). ER regulates functional and structural changes in neural circuits in both the developing and adult nervous systems by controlling the levels of cytoplasmic free Ca^{2+} locally in growth cones and synaptic compartments. The third possibility is supported by this multiple evidence. Especially, elevated basal or agonist-stimulated intracellular Ca^{2+} levels were reported in the platelets or LB cells derived from BD

patients (Kato, 2008; Warsh et al., 2004). This might also reflect the altered Ca^{2+}-buffering capacity by ER chaperones. So and colleagues reported that there was no relationship between basal calcium levels and the impaired *XBP1* induction (So et al., 2007), which does not support this speculation. However, the sample size in their report was too small to draw a conclusion, and the basal calcium level may not be the sensitive index of altered ER calcium homeostasis in LB cells. In contrast to the findings of So et al., attenuated response was seen only for thapsigargin but not for tunicamycin in this study. However, So et al. also reported that attenuation of *XBP1* induction was more prominent for the thapsigargin stimulation (58–65% reduction) compared to tunicamycin treatment (42–54% reduction). Although the reason for this discrepancy is unknown, it is intriguing that only the response to thapsigargin, but not to tunicamycin, was impaired in BD in this study. This may also support a possibility that UPR dysfunction in BD is related to altered Ca^{2+} signalling.

Although it is unknown which mechanism plays a major role in the pathophysiology of BD, these findings suggest that impairment of UPR might be relevant to altered neural functions such as neural development and plasticity.

In the present study, controls were not interviewed by a structured interview. In addition, they were not screened for family history of mental disorders. Thus, the results should be treated with caution.

In summary, altered UPR may play a role in the pathophysiology of BD. Together with pharmacological studies suggesting that lithium and valproic acid up-regulate ER chaperones, UPR might be a potential therapeutic target for BD.

Note

Supplementary material accompanies this paper on the Journal's website (http://journals.cambridge.org).

Acknowledgements

This work was supported by grants to the Laboratory for Molecular Dynamics of Mental Disorders, RIKEN BSI, a Grant-in-Aid from the Japanese Ministry of Health and Labour, and a Grant-in-Aid from the Japanese Ministry of Education, Culture, Sports, Science and Technology.

Statement of Interest

None.

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PLoS one

Valproate, a Mood Stabilizer, Induces WFS1 Expression and Modulates Its Interaction with ER Stress Protein GRP94

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Abstract

Background: Valproate is a standard treatment for bipolar disorder and a first-line mood stabilizer. The molecular mechanisms underlying its actions in bipolar disorder are unclear. It has been suggested that the action of valproate is linked to changes in gene expression and induction of endoplasmic reticulum (ER) stress-response proteins.

Principal Findings: Here we show that valproate modulates the ER stress response through the regulation of WFS1, an important component for mitigating ER stress. Therapeutic concentrations of valproate induce expression of WFS1 mRNA and activate the WFS1 promoter. In addition, WFS1 forms a complex with GRP94, an ER stress-response protein, in which valproate dose-dependently enhances its dissociation from GRP94.

Conclusions: These results suggest that the therapeutic effects of valproate in bipolar disorder may be mediated by WFS1 expression and its dissociation from GRP94.

Citation: Kakiuchi C, Ishigaki S, Oslowski CM, Fonseca SG, Kato T, et al. (2009) Valproate, a Mood Stabilizer, Induces WFS1 Expression and Modulates Its Interaction with ER Stress Protein GRP94. PLoS ONE 4(1): e4134. doi:10.1371/journal.pone.0004134

Editor: Thomas Preiss, Victor Chang Cardiac Research Institute, Australia

Received September 10, 2008; **Accepted** November 29, 2008; **Published** January 6, 2009

Funding: Research in the laboratory of F. Urano is supported by an NIH R01DK067493 grant, a grant from the Diabetes and Endocrinology Research Center at the University of Massachusetts Medical School, a Juvenile Diabetes Research Foundation Regular Research Grant, an Iacocca Foundation Grant, and an Worcester Foundation for Biomedical Research Award. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Bipolar disorder is a severe mental disorder characterized by recurrent episodes of mania and depression, affecting about 0.5–1% of the population [1]. Although the pathogenesis of bipolar disorder is unclear, it is known that mood stabilizers, such as valproate, can prevent its recurrence [2]. Valproate, a simple branched-chain fatty acid, has been used in the treatment of bipolar disorder, epilepsy, and migraine [3,4,5]. Valproate increases the level of the inhibitory neurotransmitter γ-aminobutyric acid (GABA), with acute administration causing a 15%–45% increase in GABA in the brains of rodents. Because inhibition of GABAergic signaling can cause seizures and potentiation of GABA signaling can prevent seizures, this effect of valproate on GABA levels has been proposed as a mechanism for its anticonvulsant activity [3,4,5].

The molecular mechanisms of valproate in bipolar disorder are unclear. One hypothesis is that the therapeutic effect of valproate in bipolar disorder may be mediated by changes in expression of neuroprotective genes. Valproate increases the DNA binding of activator protein 1 (AP-1), a transcription factor which is a heterodimeric protein composed of proteins belonging to the c-Fos, c-Jun, and ATF families [6,7]. This may lead to enhanced expression of AP-1 target genes that have important functions in neurons. In addition, valproate has been characterized as a histone deacetylases (HDAC) inhibitor and can regulate gene expression through epigenetic mechanisms [8]. These findings suggest an attractive possibility that valproate increases expression of multiple genes that have protective effects against bipolar disorder.

The unfolded protein response (UPR) is a gene expression program that modulates endoplasmic reticulum (ER) stress, a specific type of cell stress caused by the accumulation of misfolded proteins in the ER [9,10]. GRP94 is a component of the UPR and has a function in protein folding and degradation [11,12,13]. Genetic variations in the GRP94 gene are associated with bipolar disorder in the Japanese population [14]. High throughput proteomics analysis revealed that GRP94 interacts with WFS1 protein (Fonseca and Urano, unpublished data). WFS1 is also a component of the UPR and regulates cellular ER stress levels [15]. WFS1 was initially identified as a causative gene for Wolfram syndrome, a rare autosomal recessive disorder characterized by diabetes insipidus, diabetes mellitus, optic atrophy and deafness [16,17,18]. About 60% of patients with Wolfram syndrome have some mental disturbance such as severe depression and psychosis [19]. Importantly, even the heterozygotes who do not have Wolfram syndrome are 26-fold more likely than non-carriers to have a psychiatric hospitalization [20], and the relative risk of psychiatric hospitalization for depression was estimated to be 7.1

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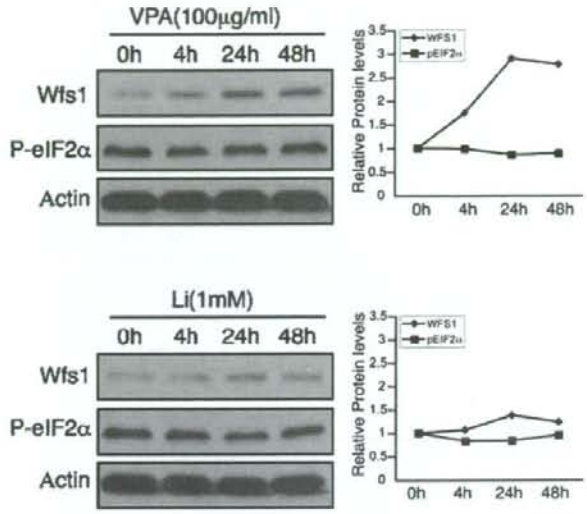

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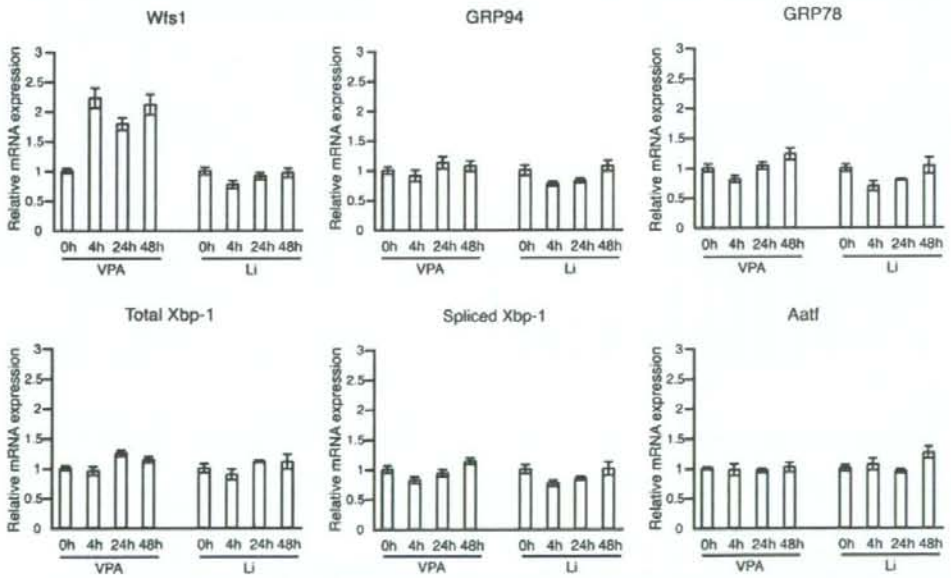

Figure 1. Valproate increases the expression of WFS1 without inducing other ER stress markers. (A) Neuro-2a cells were treated with valproate (VPA, 100ug/ml) or lithium (Li, 1mM) for 4 hr, 24 hr, and 48 hr. Expression levels of Wfs1, phospho-eIF2α (P-eIF2α) and Actin were measured by immunoblot. The relative amounts of the proteins, Wfs1 and P-eIF2α, which are adjusted by the amount of actin, are shown in the right panels. (B) Expression levels of Wfs1, GRP94, GRP78, total Xbp-1, spliced Xbp-1, and Aatf were measured by quantitative real-time PCR (n = 3; values are mean ± SD).
doi:10.1371/journal.pone.0004134.g001

[21]. These findings suggest that the modulation of ER stress by WFS1 and GRP94 may be involved in bipolar disorder.

Here we show that valproate regulates WFS1 and GRP94 in neurons. Valproate activates the WFS1 promoter and induces WFS1 mRNA expression in neuronal cells. Under normal conditions, WFS1 forms a complex with GRP94 and valproate enhances its dissociation from GRP94. Our data raise the possibility that the therapeutic effects of valproate in bipolar disorder may be mediated by the modulation of ER stress through the regulation of WFS1 and GRP94.

Results

Valproate increases the expression of WFS1 without inducing other ER stress markers

To investigate the possible involvement of valproate in WFS1 function in neurons, we first determined expression levels of WFS1 in neuronal cell lines treated with a therapeutic concentration of valproate. Valproate increased WFS1 protein expression levels in Neuro-2a cells with a peak at 24 hr (Figure 1A, upper panel). Another mood stabilizer, lithium, did not increase WFS1 expression levels significantly in these cells (Figure 1A, lower panel).

WFS1 expression is regulated by the master regulators of ER stress signaling, PERK and IRE1α, under ER stress conditions [15]. To determine whether PERK signaling is involved in WFS1 upregulation by valproate, we measured expression levels of phosphorylated eIF2α, which reflect PERK activation levels. Valproate did not increase eIF2α phosphorylation levels (Figure 1A upper panel), raising the possibility that WFS1 upregulation by valproate is not regulated by the ER stress signaling network. To test this idea, we measured mRNA expression levels of common ER stress response genes, GRP94, GRP78, total and spliced XBP-1, and AATF by real-time PCR. Figure 1B shows that expression levels of these ER stress markers did not change by valproate, indicating that valproate specifically upregulates WFS1 without activating other components of ER stress signaling.

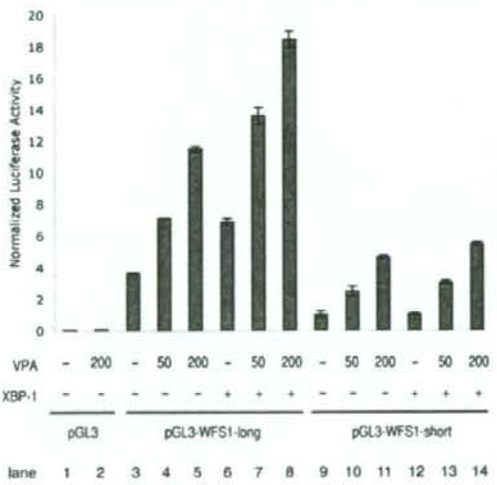

Figure 2. WFS1 promoter is activated by valproate. SH-SY5Y cells were transfected with a reporter plasmid containing 500 bases of the WFS1 promoter driving the luciferase gene (pGL3-WFS1-long), a control reporter plasmid containing only 60 bases of the WFS1 promoter (pGL3-WFS1-short), or control plasmid (pGL3) plus XBP-1 expression plasmid or control plasmid. The cells were then treated with two different concentrations of valproate, 50 μg/ml and 200 μg/ml, for 6 hr.
doi:10.1371/journal.pone.0004134.g002

It has been proposed that WFS1 mRNA expression is regulated by a 500-base-pair promoter region located upstream of its transcriptional start site [22]. We were therefore interested in determining whether this WFS1 promoter can be activated by valproate treatment. We transfected a neuronal cell line, SH-SY5Y cells, with a reporter plasmid containing 500 bases of the WFS1 promoter driving the luciferase gene or a control reporter plasmid containing only 60 bases of the WFS1 promoter, then treated these cells with two different concentrations of valproate. Valproate led to a seven fold (50 μg/ml) and a twelve fold (200 μg/ml) induction of luciferase activity (Figure 2, lanes 4 and 5). The same promoter could not be activated in non-neuronal 293T cells (data not shown). It has been postulated that XBP-1 is important in activating the WFS1 promoter in SH-SY5Y cells [22]. We therefore considered the possibility that the addition of valproate to XBP-1 expression can enhance luciferease activity. To test this idea, we co-transfected SH-SY5Y cells with XBP-1 expression plasmid along with the WFS1 reporter plasmid or the control plasmid with or without valproate treatment. As we predicted, the addition of valproate enhanced the induction of luciferase activity by XBP-1 in a dose-dependent manner (Figure 2, lanes 7 and 8). Collectively, these results indicate that valproate can strongly activate the WFS1 promoter together with XBP-1 specifically in neuronal cells.

Mood stabilizers modulate WFS1-GRP94 complex

High-throughput proteomics analysis has shown that WFS1 interacts with GRP94 (Fonseca and Urano, manuscript in preparation). To confirm this, we examined the association of WFS1 with GRP94 in Neuro-2a cells by immunoprecipitation. As we predicted, WFS1 associated with GRP94 under normal conditions (Figure 3A, lane 2, upper panels, IP).

It has been shown that valproate and lithium can modulate GRP94 expression in neurons [23], raising the possibility that this interaction can also be modulated with valproate and lithium. To test this possibility, we treated Neuro-2a cells with therapeutic concentrations of lithium or valproate for 48 hr, then examined the interaction between WFS1 and GRP94 by immunoprecipitation. The amount of GRP94 co-immunoprecipitated with WFS1 was decreased with lithium (Figure 3A, lanes 3 and 4, upper panels). The amount of WFS1 immnoprecipitated with anti-WFS1 antibody was also decreased with lithium (Figure 3A, lanes 3 and 4, lower panels, IP). Figure 3B shows that lithium treatment of Neuro-2a cells did not change the ratio between immunoprecipitated WFS1 and GRP94. These results suggest that lithium treatment may cause a conformational change of WFS1 protein, rendering the immunoprecipiation with anti-native-WFS1 antibody less efficient.

Valproate increased WFS1 expression levels in a dose-dependent manner (Figure 3A, lanes 5 and 6, lower panels, input). A parallel rise was observed in the amount of immunoprecipitated WFS1 (Figure 3A, lanes 5 and 6, lower panels, IP). Valproate decreased the ratio between immunoprecipiated WFS1 and GRP94 in a dose-dependent manner (Figure 3B), suggesting that WFS1 dissociates from GRP94 and that GRP94-free WFS1 is increased with valproate.

Discussion

Although it is well established that valproate is a standard treatment for bipolar disorder and a first-line mood stabilizer, its mechanism of action has not been fully elucidated. Our results demonstrate that valproate induces expression of WFS1 and

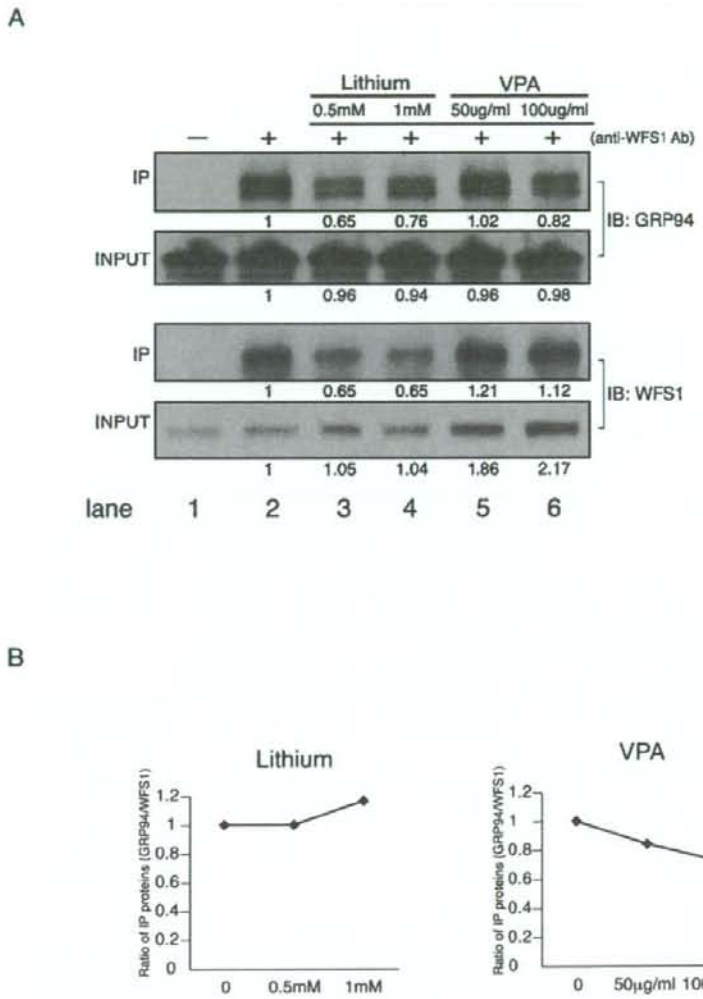

Figure 3. Mood stabilizers modulate the WFS1-GRP94 complex. (A) Neuro-2a cells were treated with lithium (Li, 0.5 mM, 1 mM), valproate (VPA, 50 ug/ml, 100 ug/ml) for 48 hr or untreated. Wfs1 was immunoprecipitated (IP) using lysates from the cells with anti-Wfs1 antibody. IP products were immunoblotted (IB) with anti-GRP94 antibody or anti-Wfs1 antibody. (B) The ratio of the relative amount of immunoprecipitaed GRP94 to that of immunoprecipitated WFS1 is shown. The X axis indicates the concentration of each drug.
doi:10.1371/journal.pone.0004134.g003

enhances its dissociation from GRP94 in neurons. We propose that the therapeutic effect of valproate is partially mediated by modulation of ER stress through the regulation of WFS1 and GRP94.

Valproate strongly activates the promoter region of WFS1 gene. We have previously shown that the minimum element for WFS1 promoter activation under ER stress conditions. The sequence of the element was similar to the one of ER stress response element (ERSE). We called it ERSE-like element [22]. The upregulation of WFS1 by valproate is probably regulated by the same element because the promoter lacking this sequence, pGL3-WFS1-short, did not respond to valproate (Figure 2). Consistent with previous results, this activation can be enhanced by co-transfection of the transcription factor XBP-1. It is possible that this activation might be indirect because our previous result indicated that XBP-1 could not directly bind to the ERSE-like element [22]. Other unknown transcription factors induced by XBP-1 or interacted with XBP-1 may have a function in the activation of WFS1 promoter.

High-throughput proteomics analysis revealed that GRP94 was one of the proteins that could interact with WFS1 in 293T cells (Fonseca and Urano, manuscript in preparation). Our data indicate that valproate enhances dissociation of WFS1 from GRP94. Considered collectively, valproate may regulate the UPR by modulating the interaction between GRP94 and WFS1.

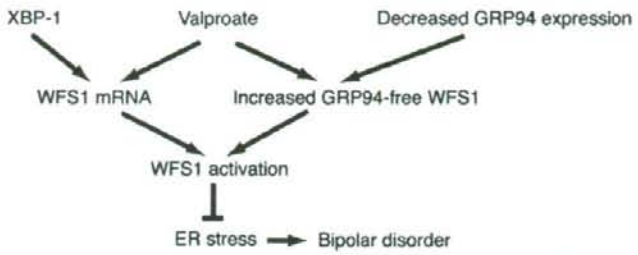

Figure 4. A speculative model of the action of valproate in the regulating of WFS1 and in the treatment of bipolar disorder.
doi:10.1371/journal.pone.0004134.g004

Genetic variations in the GRP94 gene are strongly associated with bipolar disorder in the Japanese population [14]. The protective GRP94 allele associated with bipolar disorder was related to low mRNA expression of GRP94 [14]. Downregulation of GRP94 may increase the amount of GRP94-free WFS1, leading to the enhancement of WFS1 function. Thus, the downregulation of GRP94 may have the same effect as the upregulation of WFS1. It is also possible that upregulation of WFS1 by valproate increases the ratio between GRP94-free WFS1 and GRP94-bound WFS1, leading to the activation of WFS1 (Figure 4).

GRP94 is an ER resident member of the HSP90 family of molecular chaperones. It has been shown that an HSP90 inhibitor, geldanamycin, can bind to GRP94, inhibit its function, and increase the transcription of ER molecular chaperones [24]. It would be possible that geldanamycin as well as its less toxic analogues, 17-AAG and GA, may synergize with valproate and increase its effect on WFS1 expression and modulation of the WFS1-GRP94 complex. Thus, inhibitors of GRP94 function could be a novel class of drug for bipolar disorder.

In this study, we focused on the function of valproate in WFS1 expression and its interaction with GRP94. The modulation of ER stress through the activation of WFS1 may be part of valproate's action in bipolar disorder. Our findings suggest that valproate and a compound that can reduce GRP94 expression in neurons may be a valuable treatment for patients with bipolar disorder.

Methods

Cell culture

Neuro-2a cells, SH-SY5Y, and 293T cells were maintained in DMEM with 10% fetal bovine serum.

Immunoblotting and immunoprecipitation

Cell extracts were prepared by lysis in TNE buffer (50 mM Tris-HCl pH7.5, 150 mM NaCl, 1 mM EDTA and 0.1% NP40) containing protease inhibitors and phosphatase inhibitor Cocktail 2 (SIGMA) for 15 min on ice, then the extracts were cleared by centrifuging at 12,000 g for 20 min at 4°C. Extracts were normalized for total protein (10 μg per lane), separated using 4%–20% linear gradient SDS-PAGE (Bio Rad, Hercules, CA) and electroblotted. Blots were probed with the following antibodies: anti-actin (Sigma, St. Louis, MO); anti-phospho-eIF2α, anti-GRP94 (Cell Signaling, Danvers, MA). The amount of protein was quantified using ImageJ software. For the immunoprecipitation, cells extracts were prepared by lysis in TNE buffer containing protease inhibitors for 15 min on ice. WFS1 was immunoprecipitated from the extracts with anti-WFS1 antibody, a gift from Drs. Hisamitsu Ishihara and Yoshitomo Oka (Tohoku University, Japan).

Luciferase Assay

SH-SY5Y cells were transfected with a reporter plasmid containing 500 bases of the WFS1 promoter driving the luciferase gene (pGL3-WFS1-long), a control reporter plasmid containing only 60 bases of the WFS1 promoter (pGL3-WFS1-short), or control plasmid (pGL3) plus XBP-1 expression plasmid or control plasmid using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA). 48 hrs post-transfection, the cells were treated with two different concentrations of valproate, 50 μg/ml and 200 μg/ml, for 6 hr and then lysed using a Luciferase Assay System kit (Promega, Madison, WI). The light produced from the samples was read by a plate reading luminometer, Victor X (Perkin Elmer, Waltham, MA). Each sample was read in triplicate and normalized against the signal produced from mock wells.

Real-time polymerase chain reaction

Total RNA was isolated from the cells with the RNeasy Mini Kit (Qiagen, Valencia, CA) and reverse transcribed using 1 μg of total RNA from cells with Oligo-dT primer. For the thermal cycle reaction, the iQ5 system (BioRad, Hercules, CA) was used at 95°C for 10 min, then 40 cycles at 95°C for 10 sec, and at 55°C for 30 sec. The relative amount for each transcript was calculated by a standard curve of cycle thresholds for serial dilutions of cDNA sample and normalized to the amount of actin. The polymerase chain reaction (PCR) was done in triplicate for each sample, then all experiments were repeated three times. The following sets of primers and Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) were used for real-time PCR: for mouse actin, GCAAGTGC-TTCTAGGCGGAC and AAGAAAGGGTGTAAAACGCAGC; for mouse WFS1, CCATCAACATGCTCCCGTTC and GGGT-AGGCCTCGCCATACA; for mouse GRP94, AAGAATGAAG-GAAAAACAGGACAAAA and CAAATGGAGAAGATTCC-GCC; for mouse GRP78, TTCAGCCAATTATCAGCAAAC-TCT and TTTTCTGATGTATCCTCTTCACCAGT; for mouse total XBP-1, TGGCCGGGTCTGCTGAGTCCG and GTCC-ATGGGAAGATGTTCTGG; for mouse spliced XBP-1, CTGA-GTCCGAATCAGGTGCAG and GTCCATGGGAAATGTT-CTGG; for mouse AATF, TTCTTGGCAAACCGGAGC and AGCGTCTCTGGTTCTCCTGG.

Acknowledgments

We thank Jeannette Osterloh, Kavitha Padmanabhan, and members of the Urano lab for discussions and critical reading of the manuscript.

Author Contributions

Conceived and designed the experiments: CK SI CMO TK FU. Performed the experiments: CK SI CMO SGF. Analyzed the data: CK SI CMO FU. Wrote the paper: CK SI CMO SGF TK FU.

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