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ARTICLE

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Aberrant endoplasmic reticulum stress response in lymphoblastoid cells from patients with bipolar disorder

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Abstract

Impaired endoplasmic reticulum (ER) stress response has been suggested as a possible pathophysiological mechanism of bipolar disorder (BD). The expression of ER stress-related genes, spliced form or unspliced form of XBP1, GRP78 (HSPA5), GRP94 (HSP90B1), CHOP (DDIT3), and calreticulin (CALR), were examined in lymphoblastoid cells derived from 59 patients with BD and 59 age- and sex-matched control subjects. Basal mRNA levels and induction by 4 h or 12 h of treatment with two ER stressors, thapsigargin or tunicamycin, were examined using real-time quantitative reverse transcription–polymerase chain reaction. Induction of the spliced form of XBP1 as well as total XBP1 by thapsigargin was significantly attenuated in patients with BD. Induction of GRP94 by thapsigargin was also decreased in the BD group. A haplotype of GRP94, protective against BD, exhibited significantly higher GRP94 expression upon ER stress. This report confirms and extends earlier observations of impaired ER stress response in larger samples of lymphoblastoid cell lines derived from BD patients. Altered ER stress response may play a role in the pathophysiology of BD by altering neural development and plasticity.

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Key words: Bipolar disorder, calcium homeostasis, endoplasmic reticulum stress, lymphoblastoid cells, GRP94, XBP1.

Introduction

Bipolar disorder (BD) is a severe mental illness characterized by recurrent episodes of mania and depression that affects about 1% of the population (Goodwin and Jamison, 2007). Although genetic factors contribute to the onset of this illness, genetic risk factors or causative genes have not been elucidated (Kato, 2007). Studies of action mechanisms of mood stabilizers revealed that both lithium and valproate have direct actions on neurons, including protection of neurons from cell death or enhancement of neurogenesis (Chuang, 2005; Manji and Duman, 2001). Among the findings obtained from neuroimaging studies of BD, increased incidence of white-matter hyperintensity is one of the most replicated findings (Altshuler et al., 1995); a meta-analysis showed that 9 out of 10 studies supported this finding (Videbech, 1997). Because this finding is a non-specific finding and there is no specific brain region, the infarction of which causes bipolar disorder, it would be more reasonable to assume that the hyperintensity lesion is not a causative factor but that brain cells of patients with bipolar disorder are more vulnerable to mild hypoxia. Decreased levels of *N*-acetylaspartate in the brain also suggest impaired integrity of neurons of BD patients (Stork and Renshaw, 2005). A study using olfactory neuroepithelium showed that cells derived from BD patients were more vulnerable to cell death (McCurdy et al., 2006). Studies of peripheral blood cells showed increased levels of calcium in platelets, lymphocytes and lymphoblastoid cells (reviewed by Warsh et al., 2004). These findings together suggest that BD patients have some vulnerability or impaired resilience at the cellular level (Kato, 2008; Shaltiel et al., 2007).

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However, the actual mechanism of such cellular impairment, if any, has not been elucidated. Suggested mechanisms for the effects of mood stabilizers on neurons include, among others, depletion of inositol (Williams et al., 2002), up-regulation of bcl-2 on mitochondrial outer membrane (Chen et al., 1999b), up-regulation of endoplasmic reticulum (ER) chaperones (Chen et al., 2000), and inhibition of GSK-3β (Chen et al., 1999a) effects on the extracellular signal-regulated kinase (ERK) pathway (Yuan et al., 2001). It is still unknown which of these mechanisms has the most important effect on BD and it remains to be clarified what signal cascade is impaired in the cells of BD patients.

To address this question systematically, we previously used DNA microarray analysis of lymphoblastoid (LB) cells derived from two pairs of monozygotic twins discordant with respect to BD and found down-regulated expression of *XBP1* and *GRP78* (*HSPA5*) in both affected twins (Kakiuchi et al., 2003). Because both of these genes are related to the ER stress response pathway, we focused on this pathway as a possible molecular cascade responsible for BD.

ER is a site of synthesis, folding, and modification of secretory and cell surface proteins (Yoshida, 2007), and this organelle is widely distributed throughout neurons (Murakami et al., 2007). Various cellular insults or increased demands of protein synthesis cause accumulation of unfolded proteins in the ER lumen. This condition is designated as ER stress. Unfolded protein response (UPR) is one of the adaptive responses by which cells protect themselves from ER stress. UPR consists of four signalling cascades: (1) induction of ER chaperones, e.g. GRP78 (also named HSPA5 or BiP), GRP94 (HSP90B1), calreticulin (CALR), which promotes the folding of unfolded proteins; (2) inhibition of protein synthesis; (3) induction of an ER-associated degradation pathway; and (4) induction of CHOP, a transcription factor implicated in ER stress-induced apoptosis.

XBP1 is a pivotal transcription factor that plays a crucial role in the induction of ER chaperones such as GRP78 and GRP94 (Lee et al., 2002; Yoshida et al., 2001). With ER stress, IRE1α, an endoribonuclease on ER membranes, cleaves a 26-nt fragment from an unspliced form of *XBP1* mRNA (*XBP1u*), inducing a frame shift of the open reading frame of the message. XBP1 protein encoded by the spliced mRNA (*XBP1s*) is a potent transcription factor inducing the expression of ER chaperone genes.

Several lines of evidence support the involvement of UPR dysfunction in the pathophysiology of BD. Pharmacological studies suggested that ER chaperones such as *GRP78*, *GRP94*, and *CALR* are target genes of the mood stabilizers valproate (Chen et al., 2000) and lithium (Shao et al., 2006). The antimalarial drug mefloquine, which is known to cause BD in susceptible individuals, causes ER stress (Dow et al., 2003). Serotonergic stimulation or immobilization stress activates *XBP1* splicing (Toda et al., 2006). Frequent comorbidity of mood disorders has been reported in Wolfram syndrome (Swift and Swift, 2000) caused by mutations of *WFS1* that encodes an ER stress-related protein (Fonseca et al., 2005) regulated by XBP1 (Kakiuchi et al., 2006).

Table 1. Characteristics of the subjects

		Bipolar disorder		
	Control	Total	BP I	BP II
n	59	59	43	16
Gender	34 M, 25 F	35 M, 24 F	31 M, 12 F	4 M, 12 F
Age, yr (mean ± s.d.)	47.9 ± 1.6	46.1 ± 1.6	45.5 ± 2.0	47.6 ± 3.6

We found that up-regulation of *XBP1* and *GRP78* in response to ER stress caused by thapsigargin treatment was attenuated in the cells derived from BD patients and identified that a single nucleotide polymorphism (SNP), '−116C>G' on the promoter region of *XBP1* (rs2269577) that removes the XBP1-binding sequence significantly attenuated this response (Kakiuchi et al., 2003). The attenuated *XBP1* response was partially rescued by valproate treatment. Although we initially reported that this SNP (rs2269577) was significantly associated with BD, this association was not replicated by subsequent studies (Cichon et al., 2004; Hou et al., 2004). We also reported that genetic variants of *GRP78* (*HSPA5*) (Kakiuchi et al., 2005) and *GRP94* (*HSP90B1*) (Kakiuchi et al., 2007) were associated with BD in Japanese individuals.

Recently, So and colleagues reported that LB cells derived from BD patients showed a striking decrease in the response of *XBP1* and *CHOP* to two ER stressors, thapsigargin and tunicamycin (So et al., 2007). The induction of *GRP78* did not differ from controls. To confirm and extend this finding in a relatively small number of samples (20 patients with bipolar I disorder and 10 healthy controls), we measured the induction of major UPR-related genes, spliced or unspliced forms of *XBP1*, *GRP78*, *GRP94*, *CHOP*, and *CALR* in response to ER stress, in more than 100 LB cells (obtained from 59 healthy individuals and 59 BD patients).

Methods

Subjects

The study included 59 unrelated BD patients [43 with bipolar I disorder (BD I) and 16 with bipolar II disorder (BD II) and 59 age- and sex-matched unrelated healthy controls (Table 1). Patients were diagnosed according to the DSM-IV criteria by the consensus of at least two psychiatrists. Structured interviews were not used for the diagnosis, although we began to use a structured interview, the Mini-International Neuropsychiatric Interview (MINI) (Sheehan et al., 1998) for recently recruited patients ($n=22$). Family history was obtained by an interview of the patient and available family members. Nineteen of the patients had a family history of mood disorder within first-degree relatives. Eleven of the patients had a history of psychotic features. Average age at onset was 32.2 yr (S.D. = 14.2, range 15–85). Controls were selected from students, nurses, office workers, and doctors at participating institutes along with their friends. A senior psychiatrist interviewed them and assessed them as healthy. Written informed consent was obtained from all participants. Although diabetes mellitus was not an exclusion criterion, none of the subjects had diabetes mellitus. Family history of diabetes mellitus was not assessed in the subjects. The Research Ethics Committee of RIKEN approved the study.

Cell cultures and drug treatment

Lymphocytes were separated from the peripheral blood and transformed by Epstein–Barr virus. LB cells were cultured in RPMI 1640 (Sigma; St Louis, MO, USA) containing 10% fetal bovine serum (FBS) as described previously (Kato et al., 2003). All LB cells were used after two rounds of freezing and reculturing.

For induction of ER stress, we incubated LB cells with thapsigargin (0.3 μM) or tunicamycin [2.38 μM (2 μg/ml)] for either 4 h or 12 h. We selected these time-points because 4 h is the peak of *XBP1* splicing and *XBP1u* expression, and 12 h is the peak of GRP78 expression. Thapsigargin inhibits the sarcoplasmic/ER Ca^{2+} ATPase (SERCA) leading to a rise in the cytosolic Ca^{2+} level and reduction in the ER Ca^{2+} level (Treiman et al., 1998). Tunicamycin prevents the glycosylation of newly synthesized proteins in the ER, causing the accumulation of unfolded proteins in the ER (Tordai et al., 1995). The same quantity of dimethylsulfoxide in the medium was added as vehicle control (0.5 ml in 10 ml medium). All experimental procedures and data analyses were performed blindly to the diagnosis or any other clinical variables.

RNA extraction and real-time quantitative reverse transcription–polymerase chain reaction (RT–PCR)

Total RNA was prepared from LB cells using TRIzol reagent (Invitrogen, San Diego, CA, USA), followed by DNase I (Takara Bio, Shiga, Japan) treatment to exclude genomic DNA. The SuperScriptII first-strand synthesis system with oligo(dT) (Invitrogen) was used to synthesize cDNA according to the manufacturer's instructions. Resulting cDNAs were subjected to a *Taq*Man RT–PCR assay (Applied Biosystems, Foster City, CA, USA) for the quantification of mRNA levels. The primer sequences were as follows: (sense) 5′-GGTCCAAGTTGTCCAGAATGC-3′ [spliced form of *XBP1*, (*XBP1s*)], 5′-CTCAGACTACGTGCACCTCTGC-3′ [unspliced form of *XBP1*, (*XBP1u*)]; (antisense) 5′-GCCAGTGGCCGGGTCT-3′ (*XBP1s*), 5′-AGTCAATACCGCCAGAATCCAT-3′ (*XBP1u*); (FAM-labelled probe): 5′-CCTGCACCTGCTGCGGACTCAGC-3′ (*XBP1s*), and 5′-CAGGTGCAGGCCCAGTTGTCACC-3′ (*XBP1u*). To validate the *XBP1* variant specificity of each probe set, an external control standard curve was determined by PCR with the serial dilution of pcDNA/human *XBP1s* or pcDNA/human *XBP1u* plasmid as template. To test the degree of cross-reaction, we also performed quantitative PCR with the *XBP1s* plasmid template and *XBP1u*-specific probe, or with the *XBP1u* plasmid template and *XBP1s*-specific probe. These standard curves displayed a linear relationship between C_t values and the logarithm of the input plasmid amounts (Figure S1, see Supplementary Online Appendix). Although PCR efficiency of the undesirable cross-reaction was considerably less than the specific reaction, the contribution of the cross-reaction was subtracted from the absolute value estimated by the specific reaction. All other assays were performed using the Assay-on-Demand service (Applied Biosystems); GRP94 (Assay ID, Hs00427665_g1), GRP78 (Hs00607129_gH), CALR (Hs00189032_m1), CHOP (Hs00358796_g1), and GAPDH (Hs99999905_m1). We measured the $\Delta C_t = C_t$ (each gene) $- C_t$ (*GAPDH*) for each sample in quadruplicate. The relative expression level was calculated by $2^{-\Delta C_t}$. The fold change was defined by the ratio of the relative expression level after stimulation by thapsigargin or tunicamycin to the basal relative expression level.

Genotyping of XBP1, GRP78, *and* GRP94

Genomic DNA was extracted from LB cells using standard protocols. Polymorphisms of *XBP1* (rs2269577), *GRP78* (*HSPA5*) (rs391957, rs17840761, and rs16927997), and *GRP94* (*HSP90B1*) (rs1165681 and rs17034977)

Table 2. Levels of unfolded protein response related genes at baseline

	Control ($n=59$)		Bipolar disorder ($n=59$)		
	Mean	S.D.	Mean	S.D.	p value
XBP1s	0.038	0.016	0.042	0.017	0.197
XBP1u	0.088	0.037	0.105	0.063	0.071
XBP1t	0.125	0.052	0.148	0.074	0.089
Ratio of *XBP1s/XBP1u*	0.428	0.057	0.425	0.060	0.761
GRP78	0.059	0.020	0.055	0.015	0.211
CHOP	0.00080	0.00034	0.00080	0.00021	0.527
GRP94	0.022	0.010	0.022	0.009	0.892
CALR	0.153	0.040	0.145	0.041	0.392

All genes were normalized by GAPDH.
p value: Comparison of two groups by Mann–Whitney *U* test.

were genotyped by commercially available *Taq*Man SNP genotyping assays.

In our previous paper, a haplotype (CGCTT) of five SNPs, rs1165687, rs1882019, rs17034977, rs703657, and rs2293618, in the *GRP94* gene was reported as a protective haplotype against BD. This haplotype was divided into two haplotypes based on the other upstream SNP (rs1165681), C-CGCTT and T-CGCTT. C-CGCTT was more strongly associated with BD ($p=0.0000037$, in comparison with the association with entire CGCTT, $p=0.00094$) (C. Kakiuchi et al., unpublished observations). In this study, we genotyped two SNPs, rs1165681 and rs17034977, to determine this 6-marker haplotype. This haplotype associated with BD (C-CGCTT) was named 'haplotype C-3'.

Genotyping was performed by ABI7900HT according to the protocol recommended by the manufacturer (Applied Biosystems).

Statistical analysis

The Mann–Whitney *U* test and Kruskal–Wallis test were used because these tests are robust to deviation from the normal distribution; $p<0.05$ was considered as significant. Multiple statistical analysis was not corrected because this study is based on a hypothesis and intended to replicate the previously reported findings. For the analysis of the effect of clinical variables, Spearman's coefficient of correlation was also used.

To test the effect of confounding factors, two-way analysis of covariance (two-way ANCOVA) with factors of diagnosis (d.f.=1, BD or control) and sex (d.f.=1), and a covariance of age was applied. To test the interactions between the genotype and other factors, three-way ANCOVA with the factors of genotype (d.f.=2 for *XBP1* −116C/G polymorphism and d.f.=1 for GRP94 haplotype), diagnosis (d.f.=1), sex (d.f.=1), and a covariant of age was applied. When a significant interaction between genotype and diagnosis was found, one-way ANOVA was applied to each group (BD or control). When a significant effect of genotype was found by one-way ANOVA, multiple comparison by Bonferroni correction was applied. These statistical analyses were performed by SPSS for Windows version 11.0 (SPSS Japan, Tokyo).

Results

Baseline

At baseline, no differences in the mRNA levels of *XBP1t* (total *XBP1*), *XBP1s*, *XBP1u*, *GRP94*, *GRP78*, *CALR*, and *CHOP* were found between BD patients and controls (Table 2). A significant effect of sex was found for *XBP1s*, *XBP1u*, *XBP1t*, ratio of *XBP1s/XBP1u*, *CHOP*, *and GRP94* using two-way ANOVA (Tables S1, S2, online). No other main effects and interactions were statistically significant.

XBP1

The up-regulation of ER stress-related genes in response to thapsigargin and tunicamycin was examined by real-time RT–PCR. In the presence of thapsigargin or tunicamycin, expression of *XBP1t* increased by approximately 150% after 4 h of drug application and increased expression continued, to a lesser degree, at 12 h.

The thapsigargin-induced increase of *XBP1t* at 12 h was significantly lower in the BD group than in the control group (Figure 1a, d; $p=0.019$ in

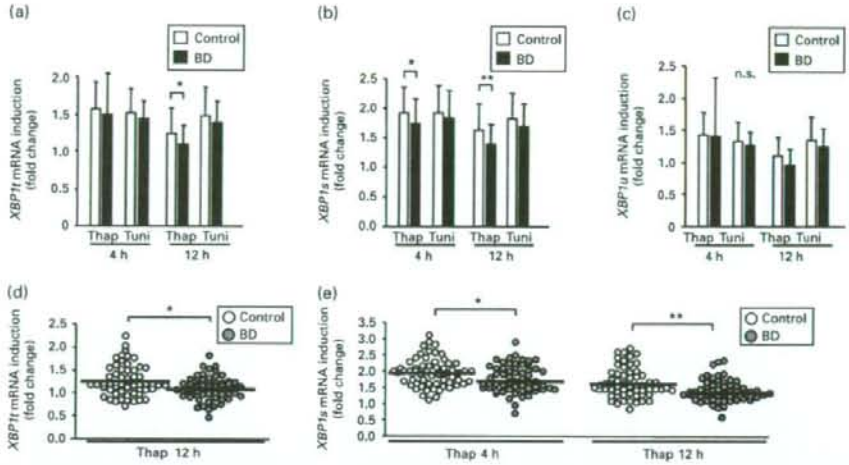

Figure 1. Altered endoplasmic reticulum (ER) stress-induced expression of *XBP1* mRNA in patients with bipolar disorder (BD). Bars represent means ± S.D. n.s., Not significant; * $p<0.05$, ** $p<0.01$ by Mann–Whitney *U* test. *XBP1t*, *XBP1* total; *XBP1s*, spliced form of *XBP1*; *XBP1u*, unspliced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

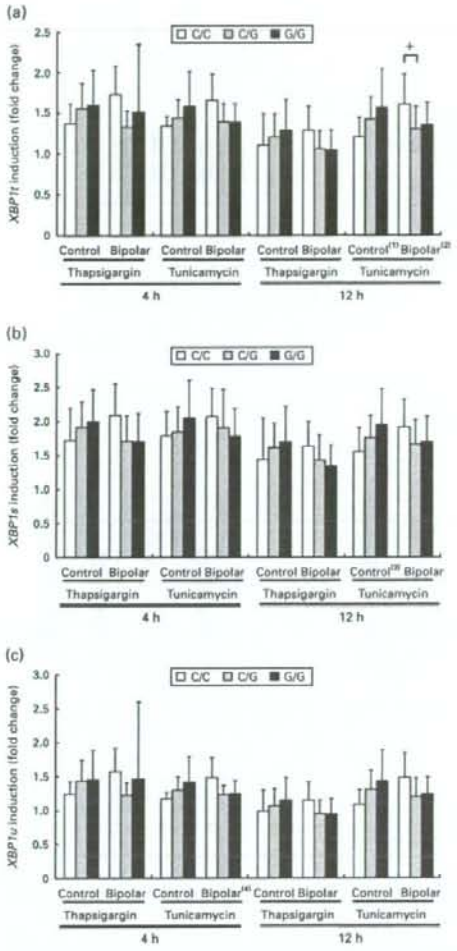

Figure 2. Effect of *XBP1* −116 polymorphism on XBP1 induction. Bars represent means ± S.D. *XBP1t*, *XBP1* total; *XBP1s*, spliced form of *XBP1*. Superior numbers indicate significance or a trend of genotype effect observed by one-way ANOVA. (1) $F=2.548$, $p=0.087$, (2) $F=2.543$, $p=0.088$, (3) $F=2.567$,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

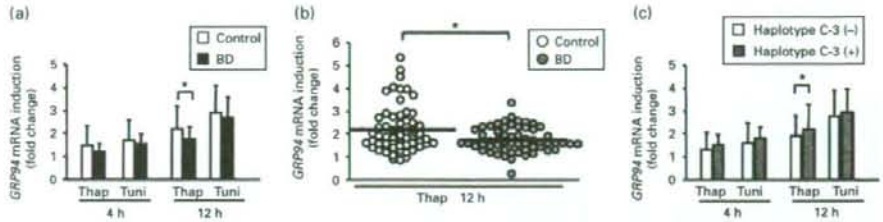

Figure 3. Induction of *GRP94* in lymphoblastoid cells. (a, b) Altered endoplasmic reticulum (ER) stress-induced expression of *GRP94* mRNA in patients with bipolar disorder (BD). (c) Difference of thapsigargin-induced *GRP94* response between the subjects with haplotype C-3 of *GRP94* ($n=17$) and those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

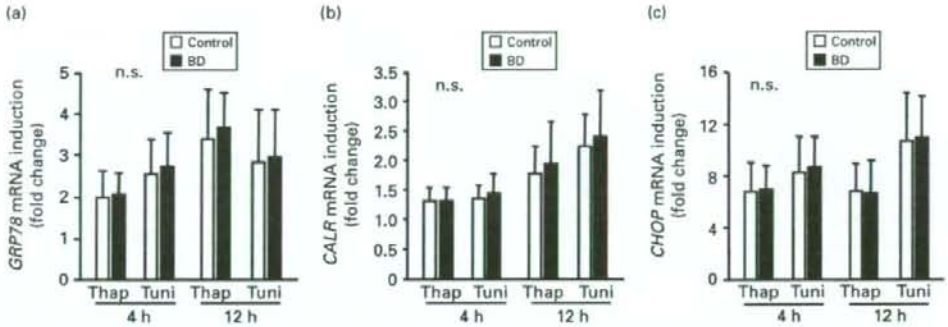

Figure 4. Induction of *GRP78*, *CALR*, and *CHOP* in lymphoblastoid cells. Bars represent means ± S.D. n.s., Not significant; BD, Bipolar disorder; Thap, thapsigargin; Tuni, tunicamycin.

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).

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Statement of Interest

None.

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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.

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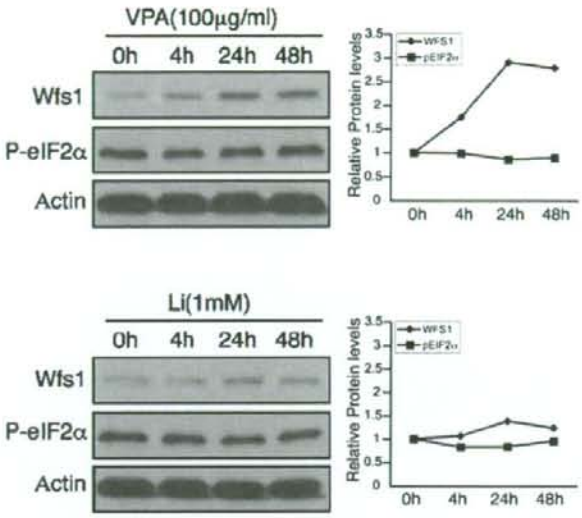
Introduction

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

A



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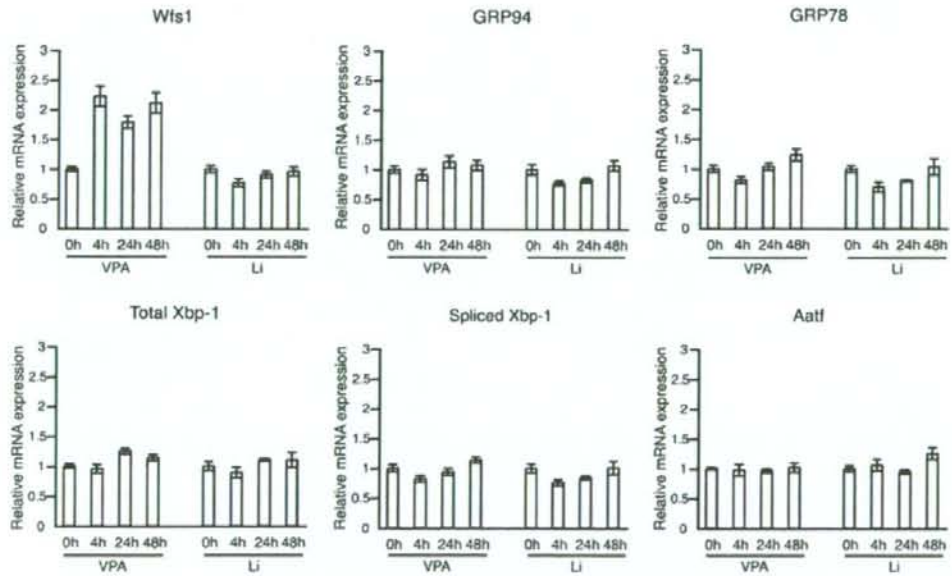

Figure 1. Valproate increases the expression of WFS1 without inducing other ER stress markers. (A) Neuro-2a cells were treated with valproate (VPA, 100ug/ml) or lithium (Li, 1mM) for 4 hr, 24 hr, and 48 hr. Expression levels of Wfs1, phospho-eIF2α (P-eIF2α) and Actin were measured by immunoblot. The 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).
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[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.

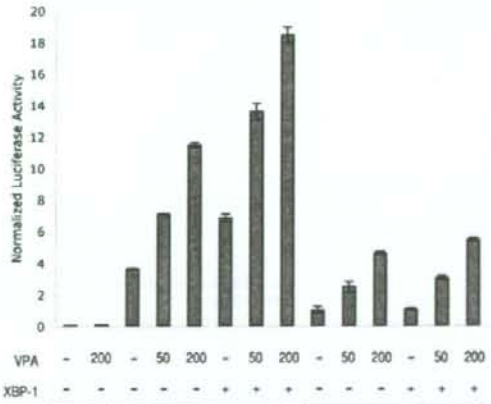

Figure 2. WFS1 promoter is activated by valproate. SH-SY5Y cells were transfected with a reporter plasmid containing 500 bases of the WFS1 promoter driving the luciferase gene (pGL3-WFS1-long), a control reporter plasmid containing only 60 bases of the WFS1 promoter (pGL3-WFS1-short), or control plasmid (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.
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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

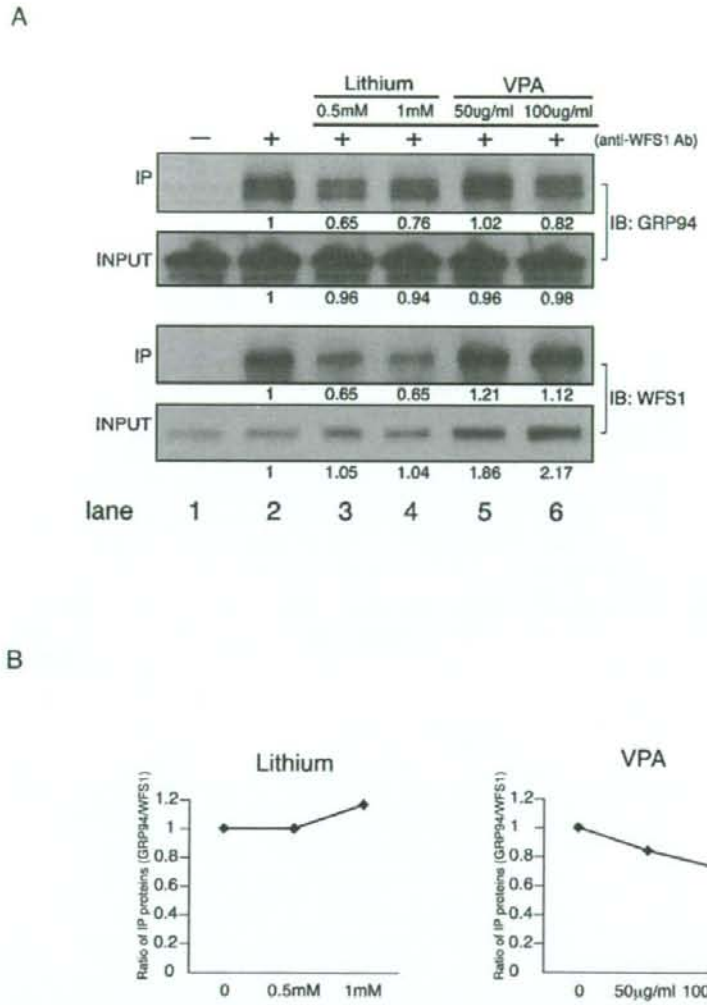

Figure 3. Mood stabilizers modulate the WFS1-GRP94 complex. (A) Neuro-2a cells were treated with lithium (Li, 0.5 mM, 1 mM), valproate (VPA, 50 ug/ml, 100 ug/ml) for 48 hr or untreated. Wfs1 was immunoprecipitated (IP) using lysates from the cells with anti-Wfs1 antibody. IP products were immunoblotted (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.

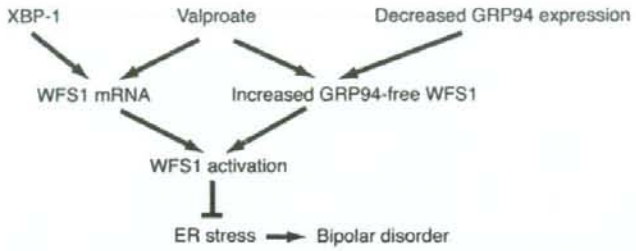

Figure 4. A speculative model of the action of valproate in the regulating of WFS1 and in the treatment of bipolar disorder.
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Genetic variations in the GRP94 gene are strongly associated with bipolar disorder in the Japanese population [14]. The protective 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.

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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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A Marked Effect of Electroconvulsive Stimulation on Behavioral Aberration of Mice with Neuron-Specific Mitochondrial DNA Defects

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Abstract

We developed transgenic (Tg) mice modeling an autosomally inherited mitochondrial disease, chronic progressive external ophthalmoplegia, patients with which sometimes have comorbid mood disorders. The mutant animals exhibited bipolar disorder-like phenotypes, such as a distorted day–night rhythm and a robust activity change with a period of 4–5 days, and the behavioral abnormalities were improved by lithium. In this study, we tested the effect of electroconvulsive stimulation (ECS) on the behavioral abnormalities of the model. Electroconvulsive therapy, which has long been used in clinical practice, provides fast-acting relief to depressive patients and drug-resistant patients. We performed long-term recordings of wheel-running activity of Tg and non-Tg mice. While recording, we administrated a train of ECS to mice, six times over two weeks or three times over a week. The treatment ameliorated the distorted day–night rhythm within three times of ECS, but it had no effect on the activity change with a period of 4–5 days in the female mice. To study the mechanism of the action, we investigated whether ECS could alter the circadian phase but found no influence on the circadian clock system. The potent and fast-acting efficacy of ECS in the mutant mice supports the predictive validity of the mice as a model of bipolar disorder. This model will be useful in developing a safe and effective alternative to lithium or electroconvulsive therapy.

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Competing Interests: RIKEN, to which all of the authors belong, has a Japanese patent (no. 2005-124412) for the mutPOLG Tg mouse as an animal model of bipolar disorder.

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Introduction

Bipolar disorder is a major mental disorder characterized by recurrent manic and depressive episodes. The involvement of genetic factors in bipolar disorder has been recognized from twin, adoption, and family studies [1]. Although linkage analyses and case-control association studies have been conducted, robustly replicated loci susceptible to bipolar disorder have not been identified [2,3]. In this context, several symptom-based animal models have been reported [4–6]. But such models hardly satisfy the construct validity; that is, these animals do not mimic aspects of the etiology.

Our group has focused on a single major gene defect with pleiotropic effects, one of which manifests bipolar disorder. Patients with chronic progressive external ophthalmoplegia (CPEO) are sometimes affected with comorbid mood disorders [7]. CPEO is a rare disease (MIM 157640) inherited in a Mendelian fashion. It is characterized by slowly but progressive ptosis and ophthalmoparesis and is associated with mitochondrial DNA (mtDNA) aberrations such as deletions, duplications, and point mutations. Mitochondrial dysfunction in bipolar disorder has been suggested by magnetic resonance spectroscopy, association studies of polymorphisms of mtDNA and nuclear genes encoding mitochondrial protein, and genome-wide gene expression analyses [8]. Recently, we generated transgenic mice as a model of bipolar disorder, in which mutant POLG (mtDNA polymerase) was expressed in a neuron-specific manner [7]. The *POLG* gene is reported to be one of the causative genes for CPEO comorbid with mood disorders [9].

The transgenic mice (mutPOLG Tg mice) harbor mtDNA defects in the brains, which show altered activities of serotonin and noradrenalin. When the mutant animals are subjected to voluntary wheel running, they show distorted day–night rhythm; they exhibit continued activity after the light is turned on (delayed activity) and untimely activity before the light is turned off (anticipatory activity). In addition, female mutPOLG Tg mice show a robust periodic activity pattern associated with the estrous cycle. These behavioral abnormalities are improved by lithium. Therefore, we propose that mutPOLG Tg mice satisfy the construct, face, and predictive validities of a model for bipolar disorder [7].

Our group has begun to pursue the cellular pathomechanism of mutPOLG Tg mice [10], as the model would accelerate medication development as well as basic research. Prior to medication development using this model, we focused on electroconvulsive therapy (ECT) in this study. ECT has long been used since before the discovery of lithium's efficacy as a mood-stabilizing agent, and ECT provides fast-acting relief to depressive patients and drug-resistant patients [11]. Here we report that electroconvulsive stimulation (ECS) markedly and immediately ameliorated the behavioral aberration of mutPOLG Tg mice.

Results

The effect of ECS on the behavioral phenotypes of mutPOLG mice

We delivered ECS six times over 2 weeks in the first experiment (Fig. 1A); this treatment schedule conformed to the standard ECT regime for patients with mood disorder. ECS seemed to improve the distorted day–night rhythm of the mutPOLG Tg mice; however, more than half of the treated mice died after ECS probably due to respiratory failure, and it became impossible to statistically analyze the effect of ECS. Therefore, in the second experiment, mice breathed in oxygen gas for about 1 min before receiving the electroshock. Oxygenation was highly effective, and mice rarely died after ECS.

Because effects of ECS were observed within the first week (after three treatments) in the first experiment, we delivered ECS three times over 1 week in the second experiment (Fig. 1B). ECS caused a reduction in wheel-running activity in the night following ECS, but this returned to the baseline levels after 1 week (Figs. 1B and 2A). Delayed and anticipatory activities, which are indicators of distorted day–night rhythm, were markedly improved (Fig. 2B and C). However, the robust periodic activity change observed in female Tg mice and day-to-day variation in wheel-running activity, one indicator of the periodic activity change, were not alleviated by ECS (Figs. 1 and 2D). Both delayed and anticipatory activities were suppressed for about 1 week after ECS (Fig. 2E), and some mice subsequently exhibited relapse of the delayed activity. In contrast, the anticipatory activity was strictly suppressed until the end of the experiment. ECS did not improve the robust periodic activity change of the female Tg mice (Fig. 1).

No influence of ECS on the phase of circadian clock

In some cases, ECS-treated mice showed regular day–night rhythm soon after the first ESC. The immediate effect of ECS allowed us to assume that ECS induced phase shifts of the circadian clock and adjusted the day–night rhythm. ECS as well as transcranial magnetic stimulation induced *c-fos* mRNA expression in the suprachiasmatic nucleus (SCN) [12], where the master circadian clock located. It is also known that *c-fos* expression is coincident with light-induced phase-shift of the circadian clock [13,14]. Thus, it might be possible that ECS improved the phenotype of the mice by directly affecting the SCN. To test this hypothesis, we examined whether ECS could alter the phase of circadian clock system. Animals were transferred and maintained in constant darkness, resulting in the free-running of circadian clock. Then we delivered a single ECS under dim red light at various times of day. We observed no phase shift caused by the ECS at any time of the day (Fig. 3).

Discussion

ECS had a dramatic effect on mutPOLG Tg mice. Delayed and anticipatory activities, indices of distorted day–night rhythm, were markedly improved by ECS (Fig. 2B, C and E). This rapid and potent effect of ECS is quite similar to that of ECT for patients with mood disorder. ECT is regarded as the most effective treatment for depression and mania [11]. According to several reports, patients with bipolar disorder respond more rapidly to ECT and require fewer treatments than unipolar patients [15,16]. In mutPOLG Tg mice, three treatments of ECS sufficiently improved both delayed and anticipatory activities. We observed the wheel-running activity of ECS-treated Tg mice for one month and found two significant outcomes. First, delayed activity suppressed by ECS gradually relapsed about 2 weeks afterward (Fig. 2E, upper), whereas anticipatory activity was completely

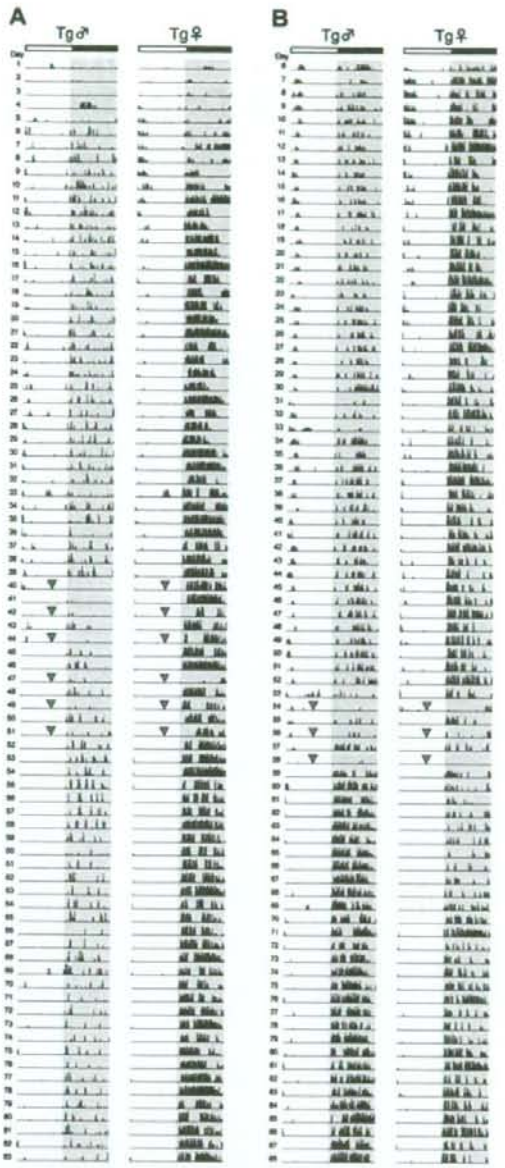

Figure 1. Wheel-running activity records of representative mice receiving ECS. Mice were delivered ECS (red triangles) six times (A) or three times (B). The light and dark periods (12:12 h) are indicated by white and gray backgrounds, respectively.
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suppressed over the 1-month period (Fig. 2E, lower). These findings suggest that there might be different mechanisms underlying delayed and anticipatory activities. Second, a robust activity change associated with the estrous cycle was not improved by ECS (Figs. 1 and 2D). Since lithium improved the periodic

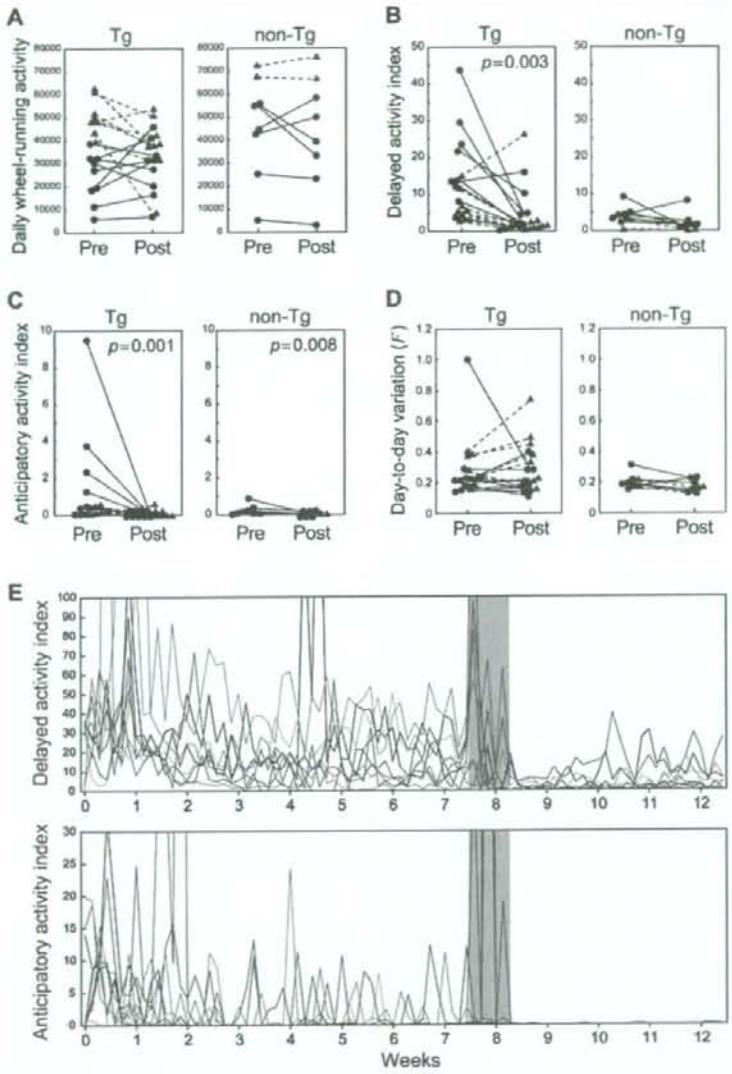

Figure 2. Effect of ECS. Mice were oxygenized and delivered ECS three times within a week (days 54, 56, and 58; shown in Fig. 1B). Wheel-running activity (A), delayed (B) and anticipatory (C) activity indices, and day-to-day variation in activity (D) were estimated on the basis of the activity levels before (Pre) and after (Post) the treatment (days 11–53 and 66–89, respectively). Values from individual male mice are indicated by closed circles connected with solid lines, and values from individual female mice are indicated by closed triangles connected with dashed lines. Delayed activity index of Tg mice and anticipatory activity index of both Tg and non-Tg mice were significantly lowered by ECS. (E) Time courses of delayed and anticipatory activity indices of male Tg mice receiving ECS. Each color of line represents different individuals ($n=9$). The week when three ECS treatments were delivered is indicated by a red background.
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activity change as well as the distorted day–night rhythm [7], we had hypothesized that the two phenotypes of the mutPOLG mice were closely related. The present result, however, indicate that these two phenotypes have different treatment response.

The potent and fast-acting efficacy of ECS in the mutant mice supports the predictive validity of the mice as a model of bipolar disorder. Although the molecular mechanism underlying the actions of lithium and ECT/ECS remains unclear, neurotrophic growth factors are suggested to be upregulated by lithium [17,18] and ECS [19–21]. This model will be useful in elucidating the action mechanism of these medications and also in developing a safe and effective alternative to lithium or ECT.