Dysregulation of the unfolded protein response (UPR) in the
dorsolateral prefrontal cortex in elderly patients with
schizophrenia

Pitna Kim, Ph.D. *,1, Madeline R. Scott, Ph.D.1, James H. Meador-Woodruff, M.D.1

1Department of Psychiatry and Behavioral Neurobiology, University of Alabama at Birmingham,
Birmingham, AL 35294, USA

Abstract

Abnormalities in protein localization, function, and post-translational modifications (PTMs) are
targets of schizophrenia (SCZ) research. As a major contributor to the synthesis, folding,
trafficking, and modification of proteins, the endoplasmic reticulum (ER) is well-positioned to
sense cellular stress. The unfolded protein response (UPR) is an evolutionarily conserved adaptive
reaction to environmental and pathological perturbation in ER function. The UPR is a highly
orchestrated and complex cellular response which is mediated through the ER chaperone protein,
BiP, three known ER transmembrane stress sensors, protein kinase RNA-like ER kinase (PERK),
activating transcription factor-6 (ATF6), inositol requiring enzyme 1α (IRE1α), and their
downstream effectors. In this study, we measured protein expression and phosphorylation states of
UPR sensor pathway proteins in the dorsolateral prefrontal cortex (DLPFC) of 22 matched pairs of
elderly SCZ and comparison subjects. We observed increased protein expression of BiP, decreased
PERK, and decreased phosphorylation of IRE1α. We also observed decreased p-JNK2 and
increased sXBP1, downstream targets of the IRE1α arm of the UPR. The disconnect between
decreased p-IRE1α and increased sXBP1 protein expression led us to measure sXbp1 mRNA. We
observed increased expression of the ratio of sXbp1/uXbp1 transcripts, suggesting that splicing of
Xbp1 mRNA by IRE1α is increased and drives upregulation of sXBP1 protein expression. These
findings suggest an abnormal pattern of UPR activity in SCZ, with specific dysregulation of the
IRE1α arm. Dysfunction of this system may lead to abnormal responses to cellular stressors and
contribute to protein processing abnormalities previously observed in SCZ.

Introduction

The onset of schizophrenia (SCZ) occurs during the neurodevelopmental period between
adolescence and adulthood, when important neural connections are formed and strengthened
to facilitate cognitive maturation1, 2. These connections use multiple neurotransmitter
systems that have been implicated in SCZ. The myriad neurotransmitter receptor abnormalities found in SCZ may suggest dysregulation of central pathways critical for modulation of these multiple systems. The endoplasmic reticulum (ER) is a major intracellular organelle that facilitates the synthesis and processing of many proteins, including those associated with neurotransmission. The ER has multiple pathways that are responsible for regulating protein quality control and responding to cellular stress. The expression of these pathways is, in part, regulated by the unfolded protein response (UPR), an evolutionarily conserved biochemical pathway that restores cellular homeostatic mechanisms. The UPR is mediated through three known ER transmembrane stress sensors, protein kinase RNA-like ER kinase (PERK), activating transcription factor-6 (ATF6), and inositol requiring enzyme 1α (IRE1α) (Figure 1). Under normal conditions, ER stress sensors are maintained in an inactive state within the ER membrane through association with the chaperone protein, glucose regulated protein 78 (Grp78), also known as BiP. In addition to the stress sensors, BiP associates with unfolded and misfolded proteins to facilitate proper protein processing. Accumulation of misfolded proteins leads to BiP dissociation from PERK, IRE1α, and ATF6, which can trigger oligomerization, autophosphorylation, and/or translocation of the UPR sensors, leading to activation of the UPR. When activated, ATF6 translocates to the Golgi, where it undergoes cleavage and is then targeted to the nucleus. In the nucleus, ATF6 acts as a transcription factor to promote UPR target gene expression. PERK and IRE1α both form homodimers in response to BiP dissociation. Once these complexes are formed they are able to autophosphorylate themselves. Autophosphorylated PERK phosphorylates eukaryotic translation initiation factor 2α (eIF2α). Phosphorylated eIF2α is no longer able to associate with the translation initiation complex, leading to decreased global protein synthesis and increased expression of activating transcription factor 4 (ATF4). ATF4 promotes expression of proteins associated with autophagy and apoptosis. Autophosphorylated IRE1α has both kinase and RNAse activity, and a major downstream effect of IRE1α activity is unconventional mRNA splicing of the transcription factor x-box binding protein 1 (XBP1). The spliced form of XBP1 (sXBP1) acts as a transcription factor and regulates expression of lipid biosynthetic enzymes, proteins responsible for ER quality control (ERQC), and ER associated degradation (ERAD). The UPR is known to regulate cell differentiation, survival, and death. While activation of the UPR is traditionally associated with increased cellular stress and misfolded proteins, constitutive activity of the UPR is also required in cells with a high secretory load, such as neurons. This leads to increased sensitivity of the brain to abnormalities in the UPR. This is reflected in studies that implicate the UPR in neurodegenerative diseases including Alzheimer’s Disease, Parkinson’s Disease, amyotrophic lateral sclerosis, Huntington’s Disease, and Creutzfeldt Jacob Disease, as well as in psychiatric disorders including bipolar disorder, major depressive disorder, and SCZ.

Previous reports demonstrating abnormal N-linked glycosylation, subcellular localization, and expression of receptor associated-proteins of both glutamatergic and GABAergic systems in SCZ brain are consistent with abnormal protein processing and subcellular targeting of neurotransmitter receptors. We propose that a potential mechanism that reconciles these findings in SCZ is abnormal synthesis and handling of receptor proteins in...
the ER. Previous work from our lab has demonstrated abnormalities in various ER-related pathways, including ERAD and glycophsatidylinositol-associated protein quality control, supporting a role for the ER in SCZ pathophysiology. The central role of the UPR in regulating protein processing led us to hypothesize that abnormalities of the UPR may be associated with ER abnormalities in SCZ brain. To test this, we measured expression of UPR sensors and associated proteins to further define UPR expression and regulation in the dorsolateral prefrontal cortex (DLPFC) in SCZ and matched comparison subjects.

Materials and Methods

Human Subjects

Samples of dorsolateral prefrontal cortex were obtained from the Mount Sinai/Bronx Veterans Administration Medical Center Department of Psychiatry Brain Collection. Assessment, consent and postmortem procedures were conducted as required by the Institutional Review Boards of Pilgrim Psychiatric Center, Mount Sinai School of Medicine and the Bronx Veterans Administration Medical Center. 22 subjects diagnosed with SCZ based on DSM-III-R criteria were pairwise matched with comparison subjects by sex, age, tissue pH, and postmortem interval (PMI) (Supplementary Table S1). Brains from subjects were removed at autopsy and sliced into 0.8-1 cm slabs in the coronal plane. Full thickness of gray matter from the cortex was blocked into 1 cm$^3$ cubes and stored at −80°C until use.

Antipsychotic Treated Rats

Male Sprague-Dawley rats were housed in pairs and treated with chronic administration of either 28.5 mg/kg haloperidol decanoate (HAL; N = 10) or vehicle (VEH; N = 10) delivered once every 3 weeks for 9 months via intramuscular injection, for a total of 12 injections. This method of drug delivery, length of treatment, and dose has been previously described. Animals were euthanized by rapid decapitation following CO$_2$ administration, frontal cortices were dissected on ice, then snap frozen and stored at −80°C until use. The Institutional Animal Care and Use Committee of the University of Alabama at Birmingham approved all procedures using these animals.

Protein Expression Studies

Tissue Preparation

For protein expression studies, tissue samples from each subjects were homogenated on ice in 5 mM Tris–HCl pH 7.5, 0.32 mM sucrose with a protease inhibitor tablet and a phosphatase inhibitor tablet (Complete Mini, EDTA-free and PhosSTOP, both from Roche Diagnostics, Mannheim, Germany) using a glass tissue grinder. Protein concentration was determined using a BCA protein assay kit (Thermo Scientific, Rockford, Illinois). After homogenization, samples were stored at −80°C until use.

Western Immunoblotting

Samples for western blot analyses were prepared with Milli-Q water and sample buffer (6X solution: 4.5% sodium dodecyl sulfate (SDS), 1.5% β-mercaptoethanol, 0.018%
bromophenol blue, and 36% glycerol in 170 mM Tris-HCl, pH 6.8) and heated at 70°C for 10 minutes. Samples were then loaded on 4-12% Bis-Tris gels (Invitrogen), subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membranes using standard semi-dry transfer methods. To reduce non-specific binding, blots were incubated in either 50% Odyssey Blocking buffer (Li-Cor) in Tris-buffered saline (TBS) or 5% bovine serum albumin in TBS for 1 hour at room temperature (RT) prior to primary antibody incubation. Conditions for probing were optimized to be within the linear range of detection for each primary antibody (Supplementary Table S2). Antibodies were stored and handled according to the manufacturer’s instructions. The specificity of commercially available antibodies was evaluated by western blotting. Validation of the specificity of commercially available antibodies that were studied is given in Supplementary Figure F1. Tris-buffered saline (TBS) + 0.1% Tween-20 was used for wash steps before and after a 1 hour incubation at RT with the appropriate secondary antibody, and membranes rinsed with Milli-Q water prior to scanning. Membranes were scanned using a LiCor Odyssey scanner at a resolution of 169 µm and intensity level of 5.

**Xbp1 mRNA Analysis**

**Total RNA isolation**

RNA was isolated using a RNeasy Mini Kit (cat# 74106 Applied Qiagen ® ). For each subject, RNA concentration was determined by spectrophotometry (ND1000, Thermo Fischer Scientific, Inc.) at 260nm and equal amounts of RNA were used to synthesize cDNA using a high capacity cDNA Reverse Transcription Kit (cat# 4368813, Applied Biosystems ® ). RIN values of all samples were determined. Over 80% of samples had RINs ≥6, an integrity value previously reported suitable for RT-qPCR studies in brain tissue53.

**Electrophoretic analysis of Xbp1 mRNA splicing**

PCR was performed with Platinum™ Green Hot Start PCR Master Mix (cat#13001012, Thermo Fischer Scientific, Inc), according to the manufacturer’s recommendations. In brief, 1 μM of each primer was mixed with 12.5 μM of Platinum™ Green Hot Start PCR Master Mix, 5 μl of Platinum GC Enhancer and 5 μl of cDNA. To prepare samples for analysis of sXbp1 mRNA, we treated the samples with PstI. Under unmodified conditions, the amount of uXbp1 is much greater than Xbp1. The section removed by IRE1α-mediated splicing is 26 bp, a small enough difference that the excess of uXbp1 masks detection of the spliced form. Therefore, we used PstI, a restriction endonuclease that cleaves Xbp1 at a site only contained within the unspliced form, to specifically digest uXbp1 and unmask sXbp1. Samples (25 μl) were incubated with PstI (1μl) overnight at 37 °C, followed by preamplification of Xbp1 transcript products using Xbp1 primer sets (forward: 5′- TTACGAGAGAAAACCTCATGG-3’/reverse: 5′-TCTACCAAGGACCCAGT-3’). Samples were then resolved on 2% agarose gels containing ethidium bromide at 60 mV for 1.5hr before visualizing by UV light. Images were collected with Gel Doc™ XR+ Gel Documentation System (Bio-Rad Laboratories, USA) and the identity of the PCR products obtained with the Xbp1 primers was verified by DNA sequencing.
Data and Statistical Analysis

Image Studio Lite Version 4.0.21 (LiCor, Lincoln, NE, USA) was used to determine integrated intensity values from both western blots and agarose gels. All samples were run in duplicate, and after intralane normalization these values were averaged. For proteins, integrated intensity values were normalized to intralane values of Valosin Containing Protein (VCP), which has been reported to not be differentially expressed in SCZ brain\(^{54}\). Expression of phosphorylated forms of proteins was normalized to the expression of their total forms. The relative amount of Xbp1 mRNA splicing was defined as the ratio of sXbp1 to uXbp1 mRNA levels. Given that many dependent variables were not normally distributed, primary analyses were performed the non-parametric Wilcoxon matched-pairs signed rank test. To determine the effect of potential covariates, Pearson or Spearman rank correlations between protein expression and subject age, tissue pH, and PMI were performed for all dependent measures. Prior to these correlation analyses, D’Agostino & Pearson omnibus normality tests were performed to determine if data were normally distributed. If a significant Pearson correlation was observed, an ANCOVA was then used to determine the effect of diagnosis when accounting for the relevant covariates. Alternatively, if a significant Spearman correlation was observed, a non-parametric ANCOVA using the Quade method was performed. All statistical analyses were performed in GraphPad Prism 6 (La Jolla, CA, USA) or STATISTICA version 7 (StatSoft, Inc, Tulsa, OK, USA) with \(\alpha = 0.05\).

Results

**UPR sensor expression and activation is abnormal in schizophrenia**

We measured expression of BiP, the three UPR stress sensors, PERK, IRE1\(\alpha\), and ATF6, and their corresponding activated states, p-PERK, p-IRE1\(\alpha\), and cl-ATF6. We observed increased in SCZ BiP expression (\(W = 235, p < 0.0001\)), decreased PERK expression (\(W = -195, p = 0.0008\)), and no change in IRE1\(\alpha\) or ATF6 expression (Figure 2, Table 1). Additionally, p-IRE1\(\alpha\) expression (\(W = -139, p = 0.0229\)) was decreased, while p-PERK and cl-ATF6 were unchanged (Figure 2, Table 1). Analysis of covariates demonstrated a positive correlation between PERK expression and age (\(r = 0.40, p = 0.007\)), and a follow-up ANCOVA indicated that when controlling for age, there is no longer a significant effect of diagnosis, suggesting that changes in PERK expression between the subject groups are driven by differences in age rather than illness. Additionally, no difference in expression of BiP, PERK, or p-IRE1\(\alpha\) was observed in rats treated with haloperidol (Figure 2).

**Expression and activation of UPR effectors suggests abnormal activation of the IRE1\(\alpha\) pathway**

In addition to the UPR sensors, we measured expression and activation of UPR downstream effectors. From the PERK pathway, we measured expression of eIF2\(\alpha\), p-eIF2\(\alpha\), and ATF4, and found no differences between comparison and SCZ subjects. In the IRE1\(\alpha\) pathway, we measured expression of both the spliced and unspliced forms of XBP1, TRAF2, and both the expression and phosphorylation of ASK1, JNK1, and JNK2. We found a decrease in SCZ of both sXBP1 (\(W = 173, p = 0.004\)) and the ratio of sXBP1/uXBP1 (\(W = 143, p = 0.02\)). Additionally, both phosphorylation of JNK2 (\(W = -165, p = 0.006\)) and the ratio of p-JNK2/JNK2 (\(W = -177, p = 0.003\)) was decreased in SCZ (Figure 3, Table 1). No changes in
uXBP1, TRAF2, ASK1, or JNK1 expression were observed, nor was there abnormal phosphorylation of ASK1 or JNK1. None of these dependent measures were significantly correlated with age, pH, or PMI, and were not different between rats treated with vehicle or haloperidol (Figure 3).

**Xbp1 mRNA Splicing is increased in schizophrenia**

We found decreased p-IRE1α and increased sXBP1 protein expression. Activation of IRE1α leads to an unconventional post-transcriptional splicing of uXbp1 mRNA to produce a spliced form of sXbp1 mRNA that is translated as sXBP1 protein\(^{55, 56}\). To determine at what stage the disconnect between IRE1α activation, as measured by p-IRE1α expression, and XBP1 splicing, as measured by sXBP1 protein expression, occurs, we measured mRNA expression of different forms of Xbp1. Similar to protein expression, we found increased expression of sXbp1/uXbp1 mRNA \((W = 169, p = 0.005)\) in SCZ (Figure 4, Table 1).

**Discussion**

In this study, we investigated the UPR through protein and activity-marker expression levels in the DLPFC in SCZ. Of the initial stress sensors of the UPR, we observed increased expression of BiP, decreased expression of PERK, and decreased phosphorylated IRE1α. We also measured downstream effectors of both the PERK and IRE1α arms of the UPR. While no change in PERK substrates was found, we observed decreased p-JNK2 and increased sXBP1, downstream targets of the IRE1α arm of the UPR. The disconnect between p-IRE1α, a marker of IRE1α activation, and sXBP1 protein expression was surprising and led us to measure sXbp1 mRNA directly. We observed increased expression of the ratio of sXbp1/uXbp1, suggesting that splicing of Xbp1 mRNA by IRE1α itself is increased and drives upregulation of sXBP1 protein expression rather than a change in sXBP1 degradation or translation. These findings suggest abnormal expression and regulation of the UPR and its downstream targets in SCZ.

These findings support a growing body of evidence for a fundamental deficit in ER protein processing pathways. Increased protein expression of BiP, phosphorylation of IRE1α and PERK, and cleavage of ATF6 are commonly used markers of UPR activity\(^{57}\). BiP is a molecular chaperone highly enriched in the ER, and has been found to be dysregulated in several proteomic studies in SCZ\(^{58-61}\). Within the lumen of the ER, BiP primarily interacts with unfolded substrates and protein folding machinery, and appears to have an active role in facilitating protein folding\(^{62}\). Cellular stress can lead to increased BiP expression, and increased expression of BiP protein has become a marker for ER stress\(^{11, 15, 57, 63}\). In this study, we observed increased BiP expression, suggesting a role for cellular stress in SCZ. This is consistent with previous reports that have demonstrated markers of increased oxidative stress, such as increased lipid peroxidation, reactive oxygen species (ROS), and decreased antioxidant levels in postmortem brain in SCZ\(^{64-67}\).

In contrast to upregulation of BiP, we also observed decreased phosphorylation of IRE1α in SCZ. Of the three sensor arms, the IRE1α pathway is the most evolutionarily conserved signaling branch\(^{2468}\). IRE1α has both serine/threonine kinase and endoribonuclease (RNase) activities, which are activated upon dissociation of BiP and subsequent dimerization of and
autophosphorylation in response to ER stress, Phosphorylation of IRE1α attracts TRAF2, which in turn promotes association of ASK1. The formation of this complex allows for IRE1α to phosphorylate ASK1, which then phosphorylates JNK. JNK is known to have three distinct isoforms, JNK1, JNK2, and JNK3, and JNK½ both upregulate proteins associated with apoptosis. Consistent with reduced p-IRE1α, phosphorylation of JNK2 was also decreased in SCZ. P-ASK1 was not significantly decreased. Decreased p-IRE1α and p-JNK2 suggests that the IRE1α-ASK1-JNK signaling pathway is downregulated, and that apoptotic processes may be attenuated in SCZ. This would be consistent with and may provide a mechanistic explanation for previous observations that there is no evidence of increased cell death despite dysregulation of cellular homeostasis in the brain in SCZ. Phosphorylation of JNK has also been observed in the ACC of SCZ subjects, supporting a more widespread role for JNK signaling downregulation in this illness.

A unique target of IRE1α RNase activity is Xbp1 mRNA, from which IRE1α removes a 26-nucleotide intron, resulting in a translational frameshift and the sXBP1 production of the spliced form of Xbp1. sXBP1 acts as a transcription factor and promotes expression of the ER translocon, ER chaperones, proteins that facilitate ERAD, glycosylation enzymes, and phospholipids. Upregulation of these pathways increases resilience of the cell to ER stress, with chaperones and glycosylation enzymes facilitating increased demand for protein folding, ERAD components clearing misfolded proteins, and phospholipids allowing for the expansion of the ER membrane during ER stress. Despite observing decreased p-IRE1α in SCZ, we observed upregulation of both sXbp1 mRNA and protein expression, suggesting upregulation of sXBP1-dependent processes. Supporting this, ERQC and ERAD components have been shown to be upregulated in the DLPFC in SCZ.

Additionally, our lab has previously reported abnormal immature N-linked glycosylation, an ER-specific protein modification, of components of the glutamate and GABA systems in the frontal cortex in SCZ. Proper regulation of the IRE1- XBP1 pathway is particularly important for neural development, and sXBP1 has been shown to specifically impact neurotransmitter systems in a developmental-dependent manner. In Xbp1−/− neurons, Hayashi et al. observed inhibition of BDNF-induced expression of GABAergic markers. In C. elegans, mutants lacking either ire-1 or xbp-1 demonstrated development-dependent inhibition of glutamate receptor trafficking. In SCZ, reduced expression of GABAergic markers has been repeatedly observed, and dysfunction in glutamatergic signaling is believed to be a key component of the illness. As a central regulator of the processes impacting these systems, sXBP1-dependent transcription may contribute to developmental deficits and neurotransmitter dysfunction.

The kinase and RNase activity of IRE1α have been shown to be independently regulated, with certain conformations promoting high kinase activity, others promoting high RNase activity, and some resulting in a combination of the two. A potential mechanism to explain the disconnect between downregulation of p-IRE1α and upregulation of Xbp1 splicing is a selective downregulation of high-kinase IRE1α complexes and preservation of oligomeric complexes with low kinase activity and high RNase activity. Alternatively, Hsp72 has been shown to bind to IRE1α and specifically increase production of sXbp1 without increasing JNK2 phosphorylation. This process appears to be cytoprotective. These mechanisms...
are not mutually exclusive, and further work to define IRE1α regulation is necessary to fully understand how IRE1α dynamics are impacted in SCZ.

In addition to Xbp1 splicing, IRE1α RNase activity also mediated regulated IRE1-dependent decay of mRNAs (RIDD), in which IRE1α degrades ER-localized transcripts to decrease translation and therefore reduce demand for protein folding and quality processes in the face of cellular stress\(^82,83\). Abnormal phosphorylation IRE1α and Xbp1 splicing both suggest that this system is also likely dysregulated. While we are unable to directly demonstrate the impact of IRE1α RNase activity on ER-associated transcripts in this study, this process may contribute to the disconnect between transcript and protein expression in SCZ postmortem brain \(^84,85\). In addition to dysregulation of the IRE1α pathway, we also observed decreased PERK expression in SCZ. However, this expression was correlated with age and an ANCOVA indicated that, when controlling for age, the effect of diagnosis on PERK expression is no longer significant. This suggests that what we observed is an effect of aging on the UPR. Age-related neurological disorders have been associated with UPR dysfunction, suggesting that this system is particularly susceptible to stressors during the aging \(^30–34,69,86,87\). Additionally, PERK mRNA has been specifically shown to be reduced in the hippocampus of aged rats\(^88\). This suggests that the UPR, and the PERK pathway in particular, is sensitive to aging and likely underlies an altered capacity to deal with ER stress in elderly individuals.

This study has several limitations. While our subjects were well-matched, they were elderly and the pathophysiology of subjects who are at later stages of the illness may not reflect abnormalities associated with earlier stages of the disorder. Using postmortem tissue limited us to investigating the UPR pathway in a cross-sectional fashion. It has been previously shown that constitutive UPR activity regulates distinct pathways from ER-stress induced UPR activity\(^89\). Therefore, while we can conclude that markers of constitutive UPR activity are abnormally expressed, we can only speculate on whether these pathways might respond to cellular stress differently in SCZ. Studies detailing the effects of protein and phosphorylation abnormalities on both constitutive and stress-induced UPR activity would be necessary to elucidate the mechanisms by which these abnormalities are contribute to pathophysiology of SCZ. Additionally, the majority of the subjects with SCZ were on antipsychotics for long periods of time. To address this confounding factor we measured the changes we observed in postmortem brain tissue in rats chronically treated with haloperidol, a commonly used first generation antipsychotic, and found no changes due to treatment. This suggests that antipsychotic treatment is not sufficient to induce these changes in brain, making our findings more likely to be due to the illness rather than chronic antipsychotic treatment.

In summary, we examined expression of key elements in the UPR in the DLPFC of patients with SCZ and matched comparison subjects. These findings suggest an abnormal pattern of constitutive UPR activity, with upregulation of cell-protective pathways and down-regulation of factors known to promote apoptosis. These abnormalities may contribute to aberrant protein processing and trafficking, and dysfunctional response to cellular stress in SCZ.
Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Signaling pathways from three ER transmembrane stress sensors during unfolded protein response (UPR) in schizophrenia

(A) ER stress stimulates the activation of the three ER transmembrane stress sensors, protein kinase RNA-like ER kinase (PERK), activating transcription factor-6 (ATF6), and inositol requiring enzyme 1α (IRE1α), that are involved in the unfolded protein response (UPR). (A) Under basal, unstressed conditions, the UPR sensors are present within the ER membrane. The heat shock protein BiP (Grp78) binds these receptors and prevents them from forming homodimers and from translocating out of the cell. (B) After dissociation of BiP, ATF6 translocates to the Golgi where it is cleaved and trafficked to the nucleus, while PERK and IRE1α form homodimers and autophosphorylate. Activation of the PERK pathway leads to phosphorylation of eukaryotic translation initiation factor 2α (eIF2α) and increased expression of activating transcription factor 4 (ATF4), while activation of the IRE1α pathway leads to unconventional mRNA splicing of the transcription factor x-box binding protein 1 (XBP1). Additionally, IRE1α activation recruits TNF receptor associated factor 2 (TRAF2) and results in phosphorylation of apoptosis signal-regulating kinase 1 (ASK1), which then phosphorylates the transcription factor c-Jun N-terminal kinase (JNK). ATF6, ATF4, and spliced XBP1 (sXBP1) function as transcription factors of UPR target genes, such as ER chaperones, the ER quality control system (ERQC) and ER-associated degradation (ERAD).
Figure 2. Altered expression level of UPR signaling pathway-related ER transmembrane stress sensor proteins in schizophrenia

(A-B) Protein expression of UPR signaling pathway-related proteins was measured in paired schizophrenia (SCZ) and comparison (COMP) subjects. (A) Representative western blots are shown for paired comparison and schizophrenia subjects. (B) Increased expression was observed for BiP, and decreased expression was observed for PERK, and p-IRE1α, though the ratio of p-IRE1α to total IRE1α was not significantly different. (C) BiP, PERK, p-IRE1α and the ratio of p-IRE1α to total IRE1α expression were not significantly different between rats that had received chronic haloperidol (HAL) treatment and those that were vehicle treated (VEH). Data are expressed as means ± SEM. *p < 0.05, ***p < 0.001, ****p <0.0001

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Figure 3. IRE1α downstream effectors are abnormally expressed and phosphorylated in schizophrenia

(A-B) Protein expression and phosphorylation of proteins downstream of p-IRE1α signaling was measured in paired schizophrenia (SCZ) and comparison (COMP) subjects. (A) p-IRE1α leads to splicing of Xbp1 mRNA and synthesis of the spliced form of XBP1 protein (sXBP1). Representative western blots of sXBP1 and unspliced XBP1 (uXBP1) are shown; both sXBP1 expression and the ratio of sXBP1/uXBP1 are increased in SCZ. (B) p-IRE1α also has kinase activity that leads to phosphorylation of protein in the ASK1/JNK signaling cascade. Representative western blots of proteins associated with p-IRE1α kinase activity are shown; both phosphorylation of JNK2 (p-JNK2) and the ratio of p-JNK2/JNK2 are decreased in SCZ. (C) p-JNK2, sXBP1 and the ratios found changed in SCZ were not significantly different in rats that had received chronic haloperidol (HAL) treatment when
compared to vehicle treated animals (VEH). Data are expressed as means ± SEM. *p < 0.05, **p < 0.01.
Figure 4. Unconventional Xbp1 mRNA Splicing is changed in schizophrenia

(A) Diagram illustrating the predicted mRNA products of Xbp1 and their digestion by PstI. uXbp1 (band 1) contain a PstI site that leads to cleavage into two transcripts (bands 3 & 4), although some of this population appears to be resistant to PstI digestion. The unique PstI site is removed when Xbp1 is spliced by IRE1α (sXbp1), resulting in this band (2) being unaffected by PstI digestion. (B) Representative resultant mRNA digested with PstI follow by emulsion cDNA and PCR amplification, and analyzed by 2% agarose gel electrophoresis to visualize the spliced (s) and unspliced (u) products. (C) The ratio of sXbp1/ uXbp1

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mRNA levels was increased in paired schizophrenia (SCZ) and comparison (COMP) subjects. Data are expressed as means ± SEM. **p < 0.01.
Table 1.
Quantification of differentially expressed proteins of ER transmembrane stress sensors in schizophrenia and comparison subjects

| Target Protein | Comparison | Schizophrenia | W Value | p-value |
|----------------|------------|---------------|---------|---------|
| BiP            | 1.01 (0.75-1.63) | 1.65 (1.23-5.92) | 235     | <0.0001 |

**ATF6 Pathway**

| Target Protein | Comparison | Schizophrenia | W Value | p-value |
|----------------|------------|---------------|---------|---------|
| ATF6           | 0.35 (0.23-0.80) | 0.42 (0.21-0.77) | 19      |         |
| cl-ATF6        | 0.37 (0.21-0.45) | 0.29 (0.18-0.48) | −29     |         |
| cl-ATF6/ATF6   | 0.75 (0.61-1.01) | 0.70 (0.59-0.78) | −109    |         |

**PERK Pathway**

| Target Protein | Comparison | Schizophrenia | W Value | p-value |
|----------------|------------|---------------|---------|---------|
| PERK           | 0.55 (0.47-0.77) | 0.46 (0.35-0.67) | −195    | 0.0008  |
| p-PERK         | 0.69 (0.44-1.28) | 0.88 (0.41-1.43) | −25     |         |
| p-PERK/PERK    | 1.35 (0.78-2.84) | 2.06 (0.65-3.35) | 95      |         |
| eIF2α          | 0.22 (0.07-0.57) | 0.20 (0.07-1.31) | 57      |         |
| p-eIF2α        | 2.07 (0.94-8.53) | 1.75 (0.97-8.45) | 39      |         |
| p-eIF2α/eIF2α  | 14.45 (0.90-20.75) | 11.17 (9.11-20.12) | 1      |         |
| ATF4           | 0.047 (0.030-0.064) | 0.052 (0.040-0.077) | 45      |         |

**IRE1α Pathway**

| Target Protein | Comparison | Schizophrenia | W Value | p-value |
|----------------|------------|---------------|---------|---------|
| IRE1α          | 1.35 (0.87-4.86) | 1.16 (0.98-4.17) | −25     |         |
| p-IRE1α        | 0.50 (0.25-0.91) | 0.28 (0.14-0.60) | −139    | 0.0259  |
| p-IRE1α/IRE1α  | 0.34 (0.19-0.89) | 0.20 (0.09-0.43) | −77     |         |

**IRE1α - XBPI Pathway**

| Target Protein | Comparison | Schizophrenia | W Value | p-value |
|----------------|------------|---------------|---------|---------|
| uXBPI          | 0.014 (0.008-0.215) | 0.013 (0.008-0.211) | −55     |         |
| sXBPI          | 0.020 (0.017-0.035) | 0.033 (0.020-0.045) | 173     | 0.0037  |
| sXBPI/uXBPI    | 1.62 (1.25-2.38) | 2.01 (1.58-2.59) | 143     | 0.0190  |
| sXBPI/uXBPI mRNA | 0.20 (0.14-0.34) | 0.28 (0.17-0.62) | 169     | 0.0047  |

**IRE1α - JNK Pathway**

| Target Protein | Comparison | Schizophrenia | W Value | p-value |
|----------------|------------|---------------|---------|---------|
| ASK1           | 0.28 (0.15-0.45) | 0.42 (0.19-0.65) | 29      |         |
| p-ASK1         | 0.58 (0.37-0.89) | 0.44 (0.36-0.70) | −109    |         |
| p-ASK1/ASK1    | 1.93 (1.05-3.11) | 1.23 (0.95-2.77) | −65     |         |
| JNK1           | 0.045 (0.035-0.078) | 0.052 (0.033-0.069) | −61     |         |
| p-JNK1         | 0.13 (0.07-0.21) | 0.10 (0.08-0.13) | −109    |         |
| p-JNK1/JNK1    | 2.35 (1.80-3.04) | 2.06 (1.74-2.89) | −69     |         |
| JNK2           | 0.064 (0.030-0.084) | 0.069 (0.034-0.102) | 65      |         |
| p-JNK2         | 0.13 (0.10-0.20) | 0.11 (0.08-0.14) | −165    | 0.0059  |
| p-JNK2/JNK2    | 2.40 (1.83-3.27) | 2.07 (1.29-2.55) | −177    | 0.0029  |

Data are reported as median (IQR). Data were analyzed using a Wilcoxon matched pairs signed rank test, n_{pair} = 22.