ESTROGEN RECEPTOR BETA AND AUTISM SPECTRUM DISORDER

By

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This thesis/dissertation is submitted by Amanda Crider and has been examined and approved by an appointed committee of the faculty of the Graduate School of Augusta University.

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ABSTRACT

AMANDA CRIDER
Estrogen Receptor Beta and Autism Spectrum Disorder
(Under the direction of DR. ANILKUMAR PILLAI)

Autism Spectrum Disorders are more prevalent in boys than in girls, with ratios of 4.5:1, suggesting the possible role of sex hormones in the pathophysiology of this. In addition to the extreme male brain theory on the high levels of testosterone during early development as a risk factor for ASD, a number of recent studies have shown the role of estrogens in the development of ASD.

Many studies have suggested an important role of endoplasmic reticulum (ER) stress in the pathophysiology of ASD, but the underlying mechanism(s) is not known. This thesis aims to determine A) the role of estrogen receptor beta in the development of Autism Spectrum Disorder and B) the role of ER stress in the regulation of ERβ and in the development of ASD pathophysiology.

KEY WORDS: Autism, Autism Spectrum Disorder, ASD, estrogen, estrogens, estrogen receptors, endoplasmic reticulum stress, IRE1, XBP1, transglutaminase 2,

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I. INTRODUCTION

A. Statement of the Problem and specific aims of the overall project

Autism Spectrum Disorder (ASD) is a set of neurodevelopmental disorders that affects 1 in 68 children in America and 1 in 160 people worldwide (1). Accurately identifying ASD is difficult as there are currently no biomarkers or medical tests available for diagnosis (1). ASD can only be diagnosed using behavioral testing and the behaviors can be subtle, making them difficult to identify (1, 2). There are standardized behavioral tests available to diagnose ASD, some of which include Autism Diagnostic Interview (ADI) and Autism Diagnostic Observation Schedule (ADOS) (3). These tests and scoring systems consist of standardized, semi-structured interviews and are typically performed by a pediatric psychologist or psychiatrist. The ADI interview is given to the parents of a child who is exhibiting ASD-like behavior and ADOS is the same category of test, but this test is given to the child. These tests measure quality of social interaction, communication skills, language skills, and repetitive or restricted behaviors or interests (3). These measures determine whether a child is achieving appropriate developmental milestones and if the child exhibits behaviors typical of children with ASD. Children who show deficits in any of the above categories and/or have not met major developmental milestones are considered for additional testing or are given an ASD diagnosis (2).

ASD is largely diagnosed by its behavioral manifestations, but the disorder has a clear genetic component. However, known genetic variants only account for approximately 10-20% of all cases (4). There is emerging evidence that epigenetic factors, which would be easier to target with novel therapeutics, contribute to the molecular pathology of ASD (5-6). Interestingly, a number of recent studies suggest an important role of Endoplasmic Reticulum (ER) stress in the pathophysiology of ASD (7). ER stress can alter the delicate balance of post-translational modifications occurring in the cell, which can lead to the improper production or degradation of functional proteins. ER stress and other theories on the pathophysiology of autism have been posited, but none of these adequately explain the clear gender bias. One theory that could explain the gender bias is hormonal dysregulation. It is known that ASD is significantly correlated with decreased Estrogen Receptor β (ERβ) gene expression (8), decreased aromatase levels (9), and high testosterone levels (10-13). A significant amount of literature implicates hormonal alterations in the development of ASD, but the factors that link ER stress to this dysregulation have not been elucidated.

The objective of this proposal is to determine the role of transglutaminase 2 (TG2) in the regulation of estrogen signaling in ASD. TG2 is a calcium-dependent enzyme that plays an important role in posttranslational modification of proteins. TG2 is located on the chromosome 20q11.2, a region associated with susceptibility to ASD (14). Moreover, gene expression studies have reported higher TG2 in the frontal cortex of ASD subjects (15). At the cellular level, ER stress, which has been implicated in ASD, has been shown to induce TG2 activity (7). TG2 is also known to inhibit aromatase activity and

expression (16). TG2 also has two Estrogen Response Elements (EREs) (14) and single nucleotide polymorphisms (SNPs) in the ERE binding region of TG2 can alter the sensitivity of TG2 to estrogen (17). Furthermore, it is known that ERα is a direct regulator of macrophage TG2 (18), further showing that TG2 can be regulated by estrogen receptors (ERs) at the genetic level. This body of literature supports a possible link between ER stress and hormonal regulation in autism, providing an explanation for gender differences as well as explaining a mechanism for ASD pathophysiology.

There are two major estrogen receptors, ERα and ERβ. ERα is thought to be responsible for modulating neurobiological reproductive systems and behavior (19). ERβ mediates some of the effects of estrogens on non-reproductive neurobiological behaviors including anxiety, locomotor activity, fear responses, and learning (19-21). ERα and ERβ have very specific tissue expression patterns. ERβ is the principal receptor expressed in cortex, hippocampus, and cerebellum (22). Our preliminary data shows a significant decrease in ERβ and estrogen receptor coactivators in the PFC of ASD subjects versus age- and gender-matched controls. However, we did not see a significant decrease in ERα levels, suggesting that ERβ deficits could be responsible for the core symptoms seen in ASD.

Based on the above literature and preliminary data, the specific hypothesis behind the project is: Increased TG2 in neurons induces ASD-like behavior in mice. This hypothesis will be tested through the following specific aims:

1. Specific Aim 1: To test the hypothesis that neuronal TG2 overexpression induces ASD-like behavior in mice.

2. Specific Aim 2: To test the hypothesis that TG2 overexpression inhibits estrogen signaling.

3. Specific Aim 3: To test the hypothesis that TG2 induced ASD-like phenotype in mice can be reversed by treatment with Estrogen Receptor Beta Agonist.

II. LITERATURE REVIEW

Estrogen was first isolated and characterized in 1929 by Tadeus Reichstein, Adolf Butenandt, and Edward Adelbert Doisy (24). The field of endocrinology progressed rapidly and in the 1950s, it was discovered that there was major interaction between the endocrine and central nervous system (CNS), coordinating activities of metabolic and developmental processes in response to the environment (24). In the 1960s, it was found that hormones affect transcription and translation of mRNA and protein. Estrogen was specifically implicated in transcriptional activation in the 1970s and 1980s by Pierre Chambon and Bert O'Malley (24). Along with the discovery of hormonal regulation of genes came the knowledge of hormone receptors.

Estrogen is produced centrally by the brain and peripherally by the ovaries and other tissues (25, 26). Estrogen plays different roles in each tissue and is involved in puberty, fertility, estrus cycle, brain development, and neuroprotection (27). Estrogen is produced from testosterone in the ovaries, corpus luteum, and placenta in premenopausal women (26). It can also be produced in the liver, heart, skin, adipose tissue, and brain (25-27). Estradiol is the bioactive form of estrogen, but other forms of estrogen are produced by females (estrone and estriol). Aromatase catalyzes the final step in estradiol synthesis and is widely expressed in many tissues, but many tissue-specific forms exist (28). Similar to

peripheral synthesis, brain estrogen synthesis can occur from testosterone, but it can also be produced de novo (27). In the brain, estrogen is produced in many regions including the hippocampus, cortex, cerebellum, hypothalamus, and amygdala (26). Neurons are the primary site of estrogen synthesis in the brain, but it can also be produced by astrocytes, but not microglia or oligodendrocytes (29). Estrogen synthesis and aromatase expression are involved in neural development, synaptic plasticity, and cell survival (29). The specificity of aromatase expression and therefore estradiol production in certain cell types and brain regions suggests that local production of estradiol is important for specific brain processes (27)

A number of recent studies have shown that estrogen plays a critical role in the pathophysiology of neurodevelopmental disorders. ASD is a neurodevelopmental disorder that affects 1 in 68 children in America and approximately 1% of the worldwide population. Individuals with ASD exhibit a wide range of symptoms including anxiety, depression, repetitive and obsessive behaviors, language and communication deficits, and social deficits. These symptoms occur over a spectrum of severity and can appear in a heterogeneous fashion in patients with the disorder. ASD appears to be gender specific, affecting boys 5x more often than girls, which rises to 10x more often in Asperger's syndrome, a high-functioning form of ASD. The increased risk for males to develop ASD suggests a potential role of sex hormones in the pathophysiology of this disorder.

1. Estrogen signaling and brain function

There are three known receptors for estrogen, estrogen receptor alpha (ERα), estrogen receptor beta (ERβ), and g-protein coupled receptor 30 (GPR30), which is also known as g-protein coupled ER1 (GPER). ERα (then known as ER) was discovered to specifically bind estrogen in 1986 (30, 31). In 1995, an additional estrogen receptor was cloned and named ERβ (32), explaining why some estrogen effects were still seen in ERα knockout mice. GPER is a third receptor with homology to g-protein coupled receptors, and is a membrane-bound receptor that binds estrogen and possibly other ligands (33).

ERα is responsible for modulating neurobiological reproductive systems such as sexual characteristics and puberty and other effects in the brain such as neuroprotection and anti-inflammatory effects (20). ERβ is important in modulating non-reproductive neurobiological systems that are involved in anxiety, locomotion, fear, memory, and learning (21). Figure 1 shows a schematic representation of the effects of estrogen on brain function through estrogen receptors α and β (22).

ERβ is the principally expressed estrogen receptor in cortex, hippocampus, and cerebellum (23). A number of cofactors have been shown to regulate the expression of ERα and ERβ at the transcriptional level. Some of these include: Proline, Glutamate And Leucine Rich Protein 1 (PELP1), steroid receptor coactivator-1 (SRC-1), transcriptional mediators/intermediary factor 2 (TIF2), amplified in breast 1 (AIB1), CREB binding protein (CBP), and CBP-associated factor (CAF), which are coactivators, as well as

silencing mediator for retinoid and thyroid hormone receptors (SMRT) and nuclear receptor corepressor (nCOR) which are corepressors (23, 24). Estrogen receptors can also regulate the production of proteins through genetic loci called estrogen response elements (EREs). These regions allow direct binding of estrogen receptors to the DNA at specific loci, which can alter gene expression. GPER appears to mediate many of the rapid, non-genomic actions of estrogen and typically involve regulation of membrane bound and cytoplasmic regulatory proteins. (34). GPER is expressed in the plasma membrane (35-36), ER and trans-Golgi network (37). Moreover, its plasma membrane localization is stabilized by association with scaffolding proteins containing PDZ (Dlg homologous region or (glycine-leucine-glycine-phenylalanine domain) binding domains, such as post synaptic density protein 95 and synapse associated protein 97, as well as with other g-protein coupled receptors (GPCRs) (38-40). In the brain, GPER is widely expressed in hippocampus, cortex and hypothalamus (41).

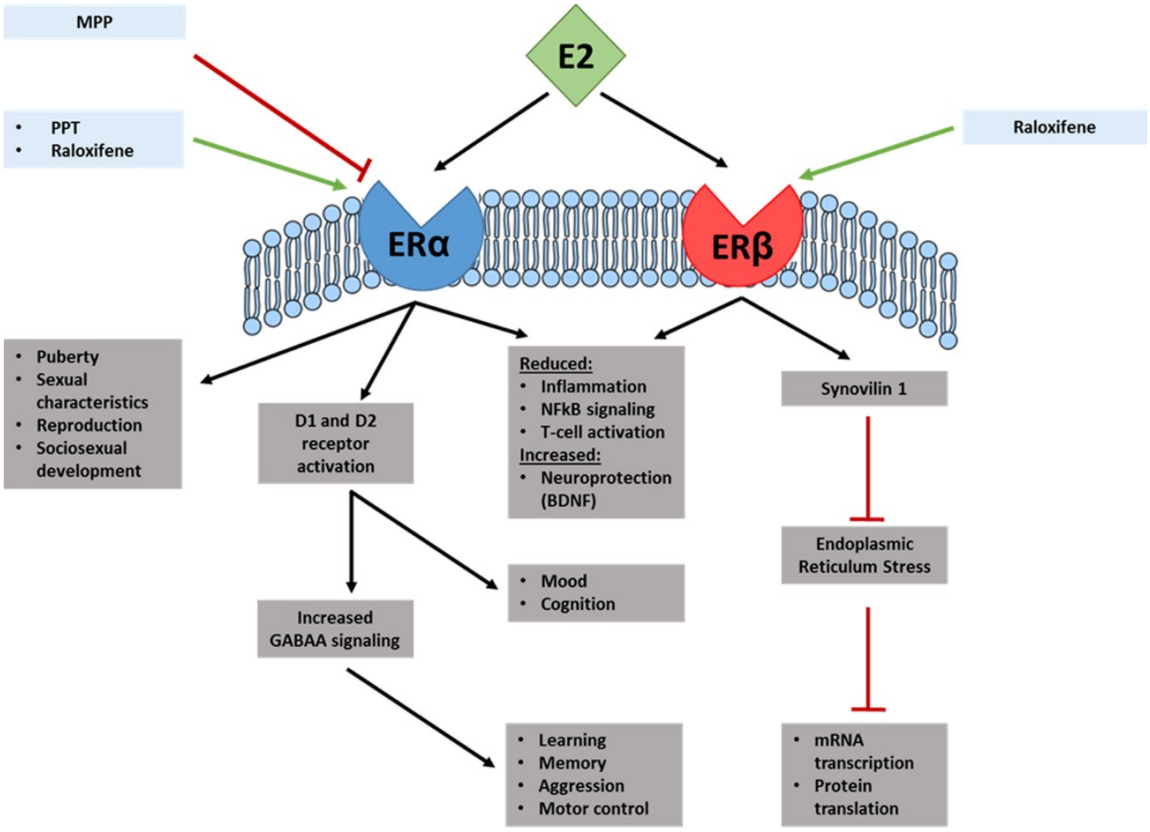

Figure 1. Schematic representation of the effects of estrogen on brain function through estrogen receptors α and β.

2. Neuroprotective effects of estrogen

Estrogen is largely thought to be neuroprotective. The bioactive form of estrogen known as 17β-estradiol (E2) has been implicated in neuroprotection, cognitive functioning, and synaptic plasticity (42). On the other hand, chronic estrogen deprivation has also been shown to increase risk of neurological disorders such as stroke, Alzheimer's disease, and Parkinson's disease (43). The neuroprotective effects of estrogen occur through genomic and non-genomic signaling, antioxidant functions, and the maintenance of neuronal adenosine triphosphate (ATP) through estrogen receptors (44). Estrogens can upregulate antiapoptotic genes and downregulate proapoptotic genes through diffusion of estradiol through the cell membrane and translocation and binding of estrogen receptors to genes in the nucleus, promoting or inhibiting transcription (43-46). This regulation seems to be largely dependent on ERα and occurs on the scale of hours (43, 46). Estrogen also exerts neuroprotective function through nongenomic signaling and localize in the plasma membrane of cortical and hippocampal neurons (43, 46). These receptors are thought to be involved in more rapid estrogen signaling including regulation of kinases, calcium signaling, and other pathways that occur on the scale of minutes to hours (43). Estradiol also protects the brain through its ability to increase cerebral blood flow, facilitation of glucose metabolism (47-48), and enhancement of electron transport chain activity to supply energy to neurons (45, 49). It is important to note that there appear to be "critical periods" or periods of effectiveness for estrogen neuroprotection (45, 50). The critical period hypothesis states that estrogen therapy must be given within a certain period of time following menopause (50). This theory follows hand in hand with the healthy cell

bias theory of estrogen which states that estrogen therapy is only effective when applied to healthy neurons (47). Animal studies have shown that estrogen can exert neuroprotective effects against ischemic damage if it is replaced immediately after injury, but not at 10 weeks post-ovariectomy (43). Also, long-term estrogen deprivation causes tissue-specific reduction in ERα levels in hippocampus, altering its sensitivity to estrogen, explaining the need for immediate estrogen replacement for neuroprotection to be effective (43). A similar reduction in ERα has been shown to occur naturally during the aging process, altering hippocampal sensitivity to estrogen (43).

Estrogen is known to promote the synthesis of neurotrophins and protects the brain against inflammation and stress. Accumulating evidence suggest that estrogen regulates the expression of brain derived neurotrophic factor (BDNF), a key molecule involved in neuronal survival, differentiation, and synaptic plasticity (51-54). ER mediated transcription has been shown to be potentiated by full length TrkB, the receptor for BDNF (55). Estrogen has also been shown to regulate BDNF mRNA and protein expression (53). In addition, estrogen treatment has been shown to restore the reduced expression of BDNF mRNA in the midbrain area of ovariectomized mice (56). Estradiol has also been shown to exert anti-inflammatory activity on activated macrophages and microglia (57-62). Estrogen is known to reduce ER stress, an inducer of inflammation, through a synovilin 1-dependent mechanism (63). It has been shown that ovariectomy is associated with changes in the peripheral immune response as evidenced by increased levels of inflammatory markers such as TNF-α, IL-1β, macrophage inflammatory protein-1, and macrophage colony-stimulating factor (65, 66). The NF-kB family of

transcription factors regulates many genes that play important roles in the function of the innate and adaptive immune systems. Moreover, estrogen-induced activation of the ER results in a reduction in the levels of nuclear DNA-binding activity of NF-kB (67), which in turn regulates the expression of inflammatory genes (68). Interestingly, treatment of ovariectomized mice with 17α-ethinylestradiol has been shown to suppress NF-KB-induced inflammatory genes (69). Moreover, treatment with estrogen modulators has been shown to reduce levels of NFkB and the activation of t-cells (70, 71). Studies conducted using ERα knockout and ERβ knockout mice have reported that both ERα and ERβ regulate proinflammatory cytokine and chemokine production in the brain through E2-dependent and E2-independent mechanisms (72). This evidence suggests anti-inflammatory action as a potential mechanism to help mediate the neuroprotective effects of estrogen.

3. Estrogen in neurodevelopment and plasticity

Estradiol is extremely important in brain development. The fetal brain is exposed to maternal estradiol, and that which is locally produced by the fetus (73). The developing brain shows high expression levels of estrogen receptors, which regulate gene expression and signal transduction (73). This expression is somewhat sex-specific and varies over time as brain development progresses (73, 74).

Estrogen's effects on learning and memory occur through rapid, non-genomic signaling pathways. Acute treatment with 17β-estradiol rapidly increased the activation of extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K)

(75, 76). Estradiol is necessary for hippocampal, frontal cortex, cerebellar, and basal forebrain-based learning and spatial memory in rats (77-79). Also, ovariectomized rats have shown impaired performance in working memory and spatial navigation tasks, and acute activation of estrogen signaling using 17β-estradiol or other potent estrogens, including synthetic estrogen receptor modulators (SERMs) could reverse the above deficits (80-84). In male mice, administration of 17β-estradiol has been shown to improve performance in inhibitory and water maze learning tasks (85). Estradiol can alter epigenetic processes within minutes to improve consolidation of hippocampal memories (86, 87). In vitro studies have shown that estradiol activation of some of the same epigenetic pathways also promotes dendritic spine remodeling, which suggests a link between spinogenesis and memory consolidation (88, 89). The knowledge that estradiol can be produced locally in the hippocampus further suggests that rapid estrogen signaling contributes to memory formation (90, 91).

Aromatase, encoded by the cyp19 gene, is the rate-limiting step in the biosynthesis of estrogens, and is widely distributed in different regions of the brain including hypothalamus, hippocampus, and cortex (92-95). It is expressed in both neurons and glial cells (90, 96). Mice deficient in aromatase show impairments in spatial reference memory (97). Interestingly, treatment with estradiol benzoate and dihydrotestosterone propionate has been shown to restore social recognition abilities in castrated aromatase KO mice (98) further suggesting the important role of estrogen in behavior.

Studies focused on specific estrogen receptor isoforms have further established the role of estrogen signaling in brain functions. Accumulating literature suggest that ERβ primarily

regulates non-reproductive components of estrogen signaling in the brain (22, 99). ERα knockout (KO) mice show severe deficits in reproduction and some alterations in learning (100). ERβ KO mice show normal sexual behavior and only slight reproductive deficits, but severe memory and learning deficits (101). Increases in ERβ enhance spatial memory while ERα facilitates sexual behavior (102). It has been shown that ERα KO, but not ERβ KO, mice showed an improvement in cognitive function following treatment with 17β-estradiol further indicating a critical role for ERβ in mediating rapid estrogenic regulation of cognitive function (103). ERβ is also important for motor learning by potentiating cerebellar plasticity and synaptogenesis (104).

A key mechanism involved in the effects of estrogen on improving cognitive function is through the regulation of synaptic structure and function (105). Seminal studies by McEwen and colleagues have shown that 17β-estradiol treatment could restore ovariectomy-induced loss of dendritic spine density in the hippocampal pyramidal neurons (106, 107). Additional studies have demonstrated that 17β-estradiol increases spine number on cortical neurons in the prefrontal cortex of ovariectomized rhesus monkeys (108, 109). The above findings from in vivo studies on the effects of 17β-estradiol on spine density are further supported by in vitro studies. It has been shown that the decrease in synapse number in response to GABA(A) receptor blockade by bicuculline was restored by estradiol treatment in hippocampal neurons (110). Similarly, aromatase inhibition using androstatrienedione has been shown to reduce dendritic spine density in cortical pyramidal neurons further suggesting an important role of estrogen in modulating spine density (111). In addition to estradiol, ERβ-selective agonist (WAY-

200070, 7-bromo-2-(4-hydroxyphenyl)-1,3-benzoxazol-5-ol) has also shown to increase the number of spines in cortical neurons (29). A recent study has reported that estradiol-induced spine changes in the dorsal hippocampus and medial prefrontal cortex are mediated through ERK and mTOR signaling mechanisms in ovariectomized mice (112). Together, these findings suggest that estrogen signaling plays a critical role in synaptic function and plasticity.

4. Estrogen signaling in autism spectrum disorder

Many studies over the years have shown that increased testosterone exposure during pregnancy due to genetic conditions or because their mothers were prescribed hormones during pregnancy (2, 39, 113, 115, 116), decreased aromatase expression (14, 117, 3), and reduced estrogen or estrogen receptor expression (14, 117) are significantly correlated with the development of ASD. Testosterone's effects on behavior and the brain have been well characterized in human and animal studies. Increased prenatal testosterone exposure appears to be significantly more correlated than postnatal exposure with the development of ASD. Increased testosterone during prenatal periods can result in social anxiety (3), reduced empathy and social development (118), and language development (115). ASD and cognitive dysfunction have been correlated with precocious puberty, which is also caused by increased testosterone load (114).

The other side of the coin of increased testosterone is reduced estrogen signaling. In rodents, sex differences in the brain are largely driven by estradiol, which is produced locally by conversion of testosterone by aromatase (119). It follows that estradiol is also

very important for proper brain development and cognition. Unlike testosterone, the effects of estrogen seem to be more profound postnatally. Reduction in estrogen signaling in adult mice through blocking aromatase or estrogen receptors has been shown to increase susceptibility to cognitive impairments due to repeated stress in rats (120). Adult females with low estrogen have also been shown to have impaired extinction and retrieval in fear extinction paradigms in rodents and humans (121, 122). Reduced estrogen receptor expression in ASD has only been established very recently (4, 117, 123, 124). Single nucleotide polymorphisms in the ERβ gene were found to be significantly associated with autism traits as measured by the Autism Spectrum Quotient and the Empathy Quotient in ASD subjects (14). In a recent study using postmortem brain samples from ASD and control subjects, we found that ERβ mRNA and protein levels are lower in the middle frontal gyrus of ASD subjects as compared to age- and gender-matched controls (117). In addition, significant reductions in aromatase (CYP19A1) mRNA and protein levels were observed in ASD subjects. CYP19A1 is enriched at synapses and localizes to presynaptic structures in neurons (29) suggesting that brain-synthesized estrogen plays an important role in neuronal function (125). The reductions in CYP19A1 could lead to impaired conversion of testosterone to estradiol resulting in increased levels of testosterone as observed in ASD subjects (39). It is known that estrogen-ER complex recruits a variety of co-regulators that result in the activation or repression of target genes by modifying chromatin structure (125). We observed significant decreases in ER co-activators such as SRC-1, CBP and P/CAF mRNA levels in ASD subjects relative to controls (117). Together, these studies suggest that a

coordinated regulation of ER and associated molecules plays an important role in ER signaling in the brain, and that this network may be impaired in subjects with ASD.

5. Autism Spectrum Disorder and Endoplasmic Reticulum stress

A number of recent studies have suggested an important role of endoplasmic reticulum (ER) stress in the pathophysiology of ASD (7, 127, 128). The ER is an intracellular organelle that is responsible for protein folding and assembly, calcium storage, and lipid and sterol biosynthesis (129). A number of pathophysiological or environmental stimuli including maternal viral infection or genetic mutations can impose stress on the ER and subsequently interrupt the protein folding process in the ER, leading to accumulation of unfolded or misfolded proteins in the ER lumen called ER stress (129). The Inositol-requiring enzyme 1 (IRE1) is an ER transmembrane sensor that activates the "Unfolded Protein Response (UPR)" to restore ER homeostasis and help the cells adapt to ER stress conditions. However, when ER stress is prolonged or the degree of ER stress is too severe, UPR signaling can initiate programmed cell death by activating stress-induced pro-apoptotic factors (130, 131). In ASD, ER stress arising from genetic variations in several synaptic genes implicated in ASD (such as neuroligin, neurexin, CNTNAP [contactin-associated protein] and CADM1 [cell-adhesion molecule 1]) have been shown to cause impaired synaptic function and signal transduction (127, 128). Moreover, relatively low levels of ER stress have been implicated in altered membrane trafficking of the synaptic functional molecules such as GABA receptors leading to ASD

pathophysiology (127). Although ER stress is implicated in ASD, the underlying mechanism(s) is not known.

6. Estrogen as a therapeutic target in neurodevelopmental disorders

The above observations suggest that an optimal balance of estrogen levels and their receptors are necessary for proper brain development and function. The profound effects of reduced estrogen signaling on ASD or schizophrenia phenotype and the potential of estrogen/estrogen receptor modulating agents to rescue behavioral deficits in rodents

suggest that estrogen signaling, more specifically ERβ agonism, is a potential therapeutic target for ASD and other neurodevelopmental disorders.

As with any hormone therapy, efficacy in males and females should outweigh the risk of sexual and other side effects. When estrogen therapy is given, men can show major side effects including feminization, gynecomastia (enlargement of the breasts), as well as other side effects such as headache and nausea (132). There are also concerns that estrogen therapies increase cancer risk and reduce fertility in men. A small study showed no increased risk of cancer in female to male transsexual men who were being administered estrogen therapy, but no large studies that are applicable to the general population have been performed (133). For women, the risks are less pronounced, but some potentially detrimental side effects are still observed. Pre and postmenopausal women who are given estrogen therapy risk the development of venous thrombosis (134).

With both men and women showing side effects from estrogen therapy, do the benefits outweigh the risks?

Estrogen has shown effectiveness in improving verbal memory loss (135), and reducing depression, aggression, anxiety, and psychotic symptoms in men with dementia (136, 137). Very few studies have been performed to explore the efficacy of estrogen or related hormone therapies in neurodevelopmental disorders. Tamoxifen has been shown to reduce mania and depression in children and adolescents with acute mania when added to lithium treatment (138). The sample sizes in the above studies are small, but the results indicate some beneficial effects of tamoxifen as an effective rapid acting antimanic agent. However, the increased risk of thromboembolic events and endometrial cancer limit the potential use of tamoxifen in long-term treatment in neurodevelopmental disorders. Although tamoxifen has mixed estrogen receptor agonist and antagonist activity depending on the target tissue, it has a number of receptor-independent effects, including inhibition of protein kinase C (PKC) (139).

ASD is a neurodevelopmental disorder that is rapidly increasing in prevalence. This disorder is complex, with each patient exhibiting slightly different manifestations of symptoms across the three major domains: social deficits, language/communication deficits, and repetitive behaviors. There are no treatments for ASD, though some drugs are used off-label to dampen individual symptoms of the disorder. These drugs have mixed efficacy and patients can show significant side effects that outweigh the benefits of the drugs. Though it is difficult, studying ASD is extremely important in order to develop

new and better treatments for patients suffering from this often debilitating disease as well as their families. This project focuses on elucidating the mechanisms involved in endoplasmic reticulum stress and ASD as well as the role of estrogen receptor β in the disorder and its efficacy as a potential therapeutic target.

III. PUBLISHED MANUSCRIPTS

1. Dysregulation of estrogen receptor beta (ERbeta), aromatase (CYP19A1) and ER co---activators in the middle frontal gyrus of autism spectrum disorder subjects

Abstract

Background

Autism spectrum disorders (ASD) are much more common in males than in females. Molecular alterations within the estrogen receptor (ER) signaling pathway may contribute to the sex difference in ASD, but the extent of such abnormalities in the brain is not known.

Methods

Postmortem middle frontal gyrus tissues (13 ASD and 13 control subjects) were used. The protein levels were examined by western blotting. The gene expression was determined by qRT-PCR.

Results

Gene expression analysis identified a 35% decrease in ERβ mRNA expression in the middle frontal gyrus of ASD subjects. In addition, a 38% reduction in aromatase (CYP19A1) mRNA expression was observed in ASD subjects. We also found significant decreases in ER co-activators that included a 34% decrease in SRC-1, a 77% decrease in

CBP, and a 52% decrease in P/CAF mRNA levels in ASD subjects relative to controls. There were no differences in the mRNA levels of TIF-2, AIB-1 (ER co-activators), ER co-repressors (SMRT and nCoR) and ERα in the middle frontal gyrus of ASD subjects as compared to controls. We observed significant correlations between ERβ, CYP19A1, and co-activators in the study subjects. Immunoblot analysis further confirmed the changes in ERβ and aromatase at the protein level in the control and ASD subjects.

Conclusions

These results, for the first time, provide the evidence of the dysregulation of ERβ and co-factors in the brain of subjects with ASD.

Background

Autism spectrum disorders (ASD) are a heterogeneous set of neurodevelopmental disorders including autism, Asperger's syndrome, childhood disintegrative disorder, and pervasive developmental disorder not otherwise specified (PDD-NOS). Autism is currently diagnosed by extensive behavioral and psychological testing and diagnosis is declared based on DSM-V characteristics. These characteristics include communication deficits, excessive dependence on routine, and obsessive tendencies showing up in early childhood. ASDs are more prevalent in boys than in girls, with ratios of 3:1 for classic autism and 10:1 for Asperger syndrome, suggesting the possible role of sex hormones in the pathophysiology of this disorder (140). It has been suggested that high levels of testosterone during early development may be a risk factor for ASDs (141, 13). The above hypothesis has been supported by a number of studies reporting an association between fetal testosterone levels and autistic features (141).

Estradiol, the most potent estrogen, is formed from testosterone by the enzyme aromatase, also known as cytochrome P450, family 19 (CYP19A1). Estrogen is neuroprotective and plays an important role in emotional responses (142) and in frontal cortical activity during cognitive task performance in humans (143). Estrogen acts through the binding to its receptor called estrogen receptor (ER). ER exists in two main forms, ERα and ERβ, which have distinct tissue expression patterns (144). ERα and ERβ are encoded by separate genes, ESR1 and ESR2, respectively, found at different chromosomal locations, and numerous mRNA splice variants exist for both receptors in both diseased and normal tissue (145). In the "classical" pathway of estrogen action, estrogen binds to ER, a ligand-activated transcription factor that regulates transcription of target genes in the nucleus by binding to estrogen response element regulatory sequences in target genes and recruiting co-regulatory proteins such as co-activators or co-repressors. The major co-regulators involved in estrogen signaling are steroid receptor co-activator 1 (SRC-1), transcriptional mediators/intermediary factor 2 (TIF-2), nuclear receptor co-repressor 1 (nCoR), CREB-binding protein (CBP), p300/CREB-binding protein-associated protein (P/CAF), amplified in breast 1 (AIB-1), and silencing mediator of retinoid and thyroid hormone receptors (SMRT) (146). Among these co-factors, SRC-1, TIF-2, CBP, AIB-1, and P/CAF are transcriptional co-activators, whereas SMRT and nCoR are transcriptional repressors. On the other hand, in the rapid or "non-genomic" pathway, activation of the membrane ER by estrogen leads to a rapid change in various intracellular signaling molecules including kinases, which in turn regulate gene transcription (147).

ERα is widely distributed in the various brain regions including amygdala-hippocampal area, periamygdaloid cortex, and posterior cortical nucleus of the brain (148). Moreover, ERα influences various neurotransmitter systems, such as dopamine, serotonin (5-HT), and norepinephrine, indicating its role in neuropsychiatric disorders (146, 145). ERβ is the principal estrogen receptor expressed in brain areas such as cerebral cortex, hippocampus, and cerebellum (146). An earlier study has found a significant association of the ERβ gene with scores on the Autism Spectrum Quotient and the Empathy Quotient in ASD subjects (147). Moreover, ERβ mediates some of the effects of estrogens on anxiety, locomotor activity, fear responses, and learning behavior (148). Although the above studies are interesting, it is still unclear whether the expression of ER is impaired in the brain of ASD subjects. In the present study, we first examined the gene expression of ERα, ERβ, CYP19A1, and the co-regulators SRC-1, TIF-2, CBP, P/CAF, SMRT, AIB-1, and nCoR in postmortem middle frontal gyrus of ASD and control subjects. The middle frontal gyrus region was selected because a number of studies from neurocognitive as well as neuroimaging studies have implicated middle frontal gyrus in the pathophysiology of ASD (149-151). Moreover, a sexual dimorphic nature has been reported in the middle frontal gyrus (152). Based on our mRNA data, we then used western blotting to examine whether the changes found at the gene level of ERβ and CYP19A1 are significant at the protein level. We hypothesized that the expression of ERs are impaired in ASD and these abnormalities involve key co-regulators involved in ER regulation.

Methods

Ethics statement

The Georgia Regents University Institutional Review Board has deemed this study exempt from full review due to the use of de-identified human postmortem brain samples, with no possibility to track back the identity of the donors. There was no involvement of animal studies in this paper.

Postmortem brain tissues

Postmortem middle frontal gyrus tissues of ASD (n = 13) and control (n = 13) subjects were received from the NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, MD, USA. Table 1 shows a detailed description

Table 1. Demographic characteristics of postmortem brain samples

Variable	Control	ASD
Age (years)	11.70 ± 1.584	11.80 ± 1.609
PMI (h)	14.46 ± 2.171	19.00 ± 2.776
Sex (F/M)	1/12	0/13
Storage (days)	4287 ± 638.7	2829 ± 397.7
RIN	5.55 ± 0.71	6.84 ± 0.53
pH	5.95 ± 0.06	6.11 ± 0.07
Manner of death	Drowning (3), Vehicle accident (4), Hanging/suicide (2), Cardiovascular complication (1), TSS (1), Multisystem failure (1), Anaphylaxis (1)	Drowning (6)
		Vehicle accident (1)
		Cardiovascular complication (2)
		Cancer (1)
		Hemorrhage (2)
		Diabetic ketoacidosis (1)
Medications*	Yes (1) (Concerta, Clonidine)	Yes (4) (Zyprexa (1), Reminyl (1), Naltrexone (1), Risperdal (2), Luvox (1), Clonidine (1), Insulin (1))
	No (12)	No (9)

ASD, Autism spectrum disorder; F, Female; M, Male; PMI, Postmortem interval; RIN, RNA integrity; TSS, Toxic shock syndrome. Values are Mean ± SE. *Note that some individuals took multiple medications.

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on the demographics of samples. The individual scores for each of the symptomatic domains, Autism Diagnostic Interview-Revised (ADI-R) were obtained from the brain bank website. The information on ADI-R was available for 9 out of 13 subjects with ASD. We did not find any significant difference in confounding variables such as PMI, refrigeration interval, age at death, RNA integrity, and brain pH between ASD and control subjects.

Immunoblotting

Brain tissue was homogenized in a tissue lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1.0% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 2 mM EDTA, 6 µM PMSF, and 1.0% Triton X-100 supplemented with protease inhibitor cocktail (Sigma). The homogenate was centrifuged at 13,000 rpm for 10 min at 4°C and the supernatant was used for protein estimation by the bicinchoninic acid method (BCA Protein Assay Kit, Sigma). Samples (30–40 µg) were subjected to SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was blocked for 1 h in PBS with Tween 20 and 5% non-fat milk or 5% BSA followed by overnight incubation with a primary antibody. The primary antibodies used were: anti-ERβ (1:2,000, Abcam; ab3577 (20, 21); anti-ERα (1:500, Santa Cruz Biotech; sc-71064); anti-aromatase (1:500, Santa Cruz Biotech; sc-14245); anti-CBP (1:200, Santa Cruz Biotech; sc-7300); anti-SRC-1 (1:500, Santa Cruz Biotech; sc-32789), or anti-P/CAF (1:500, Santa Cruz Biotech; sc-

13124). Following washing, the membranes were incubated with secondary antibody for 1 h. We used enhanced chemiluminescence detection reagent kit (Thermo Scientific) to detect the proteins. The intensity of the bands was quantified using densitometry software (Image J, NIH). The immunoblot data was corrected for corresponding glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:5,000, Cell Signaling) values. For immunoprecipitation, 300 µg of proteins were pre-cleared for 2 h with 40 µL of PureProteome Protein A and G Magnetic Beads (Millipore) and 40 µg IgG antibody (Millipore), followed by incubation overnight with the primary antibody. The immunoprecipitated proteins were subjected to immunoblotting for the detection of co-precipitated proteins.

Quantitative reverse transcriptase PCR (qRT-PCR)

Total RNA from postmortem brain tissues was isolated using a commercially available kit (SV RNA Isolation, Promega, Madison, WI, USA). qRT-PCR was performed on a MasterCycler (Eppendorf) using a SuperScript III Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen, Carlsbad, CA, USA). A typical reaction mixture of a total volume of 25 µL consisted of 0.5 µL Superscript III RT/Platinum Taq mix, 12.5 µL 2X SYBR Green Reaction Mix (includes 0.4 mM of each dNTP and 6 mM MgSO4), 12.5 pMol of each of forward or reverse primers, and 4 µL DEPC-treated water. PCR amplification was done with an initial incubation at 55°C for 1,200 sec, then at 95°C for 120 sec followed by 35 cycles of 95°C for 15 sec, 50°C for 30 sec, 72°C for 30 sec, and a final melting curve from 55°C to 95°C at 0.2°C/sec. We confirmed the primer specificity by melting curve analysis and electrophoresis of PCR products on a 2% agarose gel to

confirm the presence of a single band of the predicted size. The mRNA for genes of interest was normalized to two control genes (GAPDH and β-actin) and a geometric mean of these genes. The mRNA expression levels were quantified by the delta-delta Ct method. Primers were synthesized by Integrated DNA Technologies (Additional file 1: Table S1).

Statistical analysis

Analysis of covariance (ANCOVA) models were used to examine the differences in estrogen receptor expression between postmortem samples of people with ASD and the control sample (i.e., affection status). To examine the unique effects of affection status on estrogen receptor, age, postmortem interval, storage time, sample pH, and RNA integrity number were added to the model as covariates. Only covariates with at least small associations with an estrogen receptor expression were considered for inclusion in the ANCOVA model with the receptor signal as a dependent variable. Candidate covariates that were significantly correlated with receptor expression levels were included in the model. To maximize the observed power, non-significant covariates were removed from the model until only significant covariates remained in the model; η2 and partial η2 coefficients were computed as estimates of effect size. Exact probability (P) values of less than 5% were considered significant. Simple correlations were computed to examine the association of covariates and clinical variables with receptor expression levels. All analyses were performed using SPSS Statistics 20 software (IBM).

Results

The postmortem sample comprised 26 subjects, half of whom had a confirmed diagnosis of an ASD and half of whom were age- and gender-matched controls.

Decrease in mRNA levels of ERβ and CYP19A1, but no change in ERα mRNA in the middle frontal gyrus of ASD subjects

The mRNA levels of ERα, ERβ, and CYP19A1 in the middle frontal gyrus of control and ASD subjects were determined by qRT-PCR. Additional file 2: Table S2 depicts the correlations between the mRNA transcripts and confounding variables. An ANCOVA [between-subjects factor: affection status (ASD, Control); covariates: age, postmortem interval, storage time, sample PH] was performed in ERα data analysis. None of these covariates demonstrated significant main effects in the prediction of ERα expression. The main effect of affection status on ERα was not statistically significant (Figure 1A) [$F(1, 19) = 2.65$, $P = 0.12$, $\eta 2p = 0.123$]. We found a statistically significant main effect of affection status on ERβ mRNA levels when age, storage time, and RNA integrity number were considered in the model as covariates (Figure 1B) [$F(1, 20) = 34.10$, $P < 0.0001$, $\eta 2p = 0.630$]. None of the covariates demonstrated a significant main effect on ERβ mRNA. Subjects with ASD (estimated marginal means (EMM) = 65.85, standard error (SE) = 4.06) demonstrated much lower expression (35% decrease) of ERβ than the control group (EMM = 100.59, SE = 3.88). For CYP19A1 mRNA data analysis, age, storage time, sample pH, RNA integrity number, and postmortem interval were entered into the model as covariates. None of the covariates in the model demonstrated a significant main effect in the prediction of CYP19A1 mRNA when they were entered in the model with affection status. Affection status was a significant predictor of CYP19A1 expression

(Figure 1C) [$F(1, 17) = 27.90$, $P < 0.0001$, $\eta 2p = 0.621$]. Subjects with ASD had reduced (EMM = 62.76, SE = 4.78) CYP19A1 mRNA levels (38% decrease) relative to the control group (EMM = 101.29, SE = 4.78).

Table 2 shows the correlation of mRNA transcripts with ADI-R scores. We have adjusted the P values using the Holm-Bonferroni sequential correction for multiple comparisons. We did not find any significant correlation of ERα, ERβ, and CYP19A1 mRNA levels with any of the ADI-R scores.

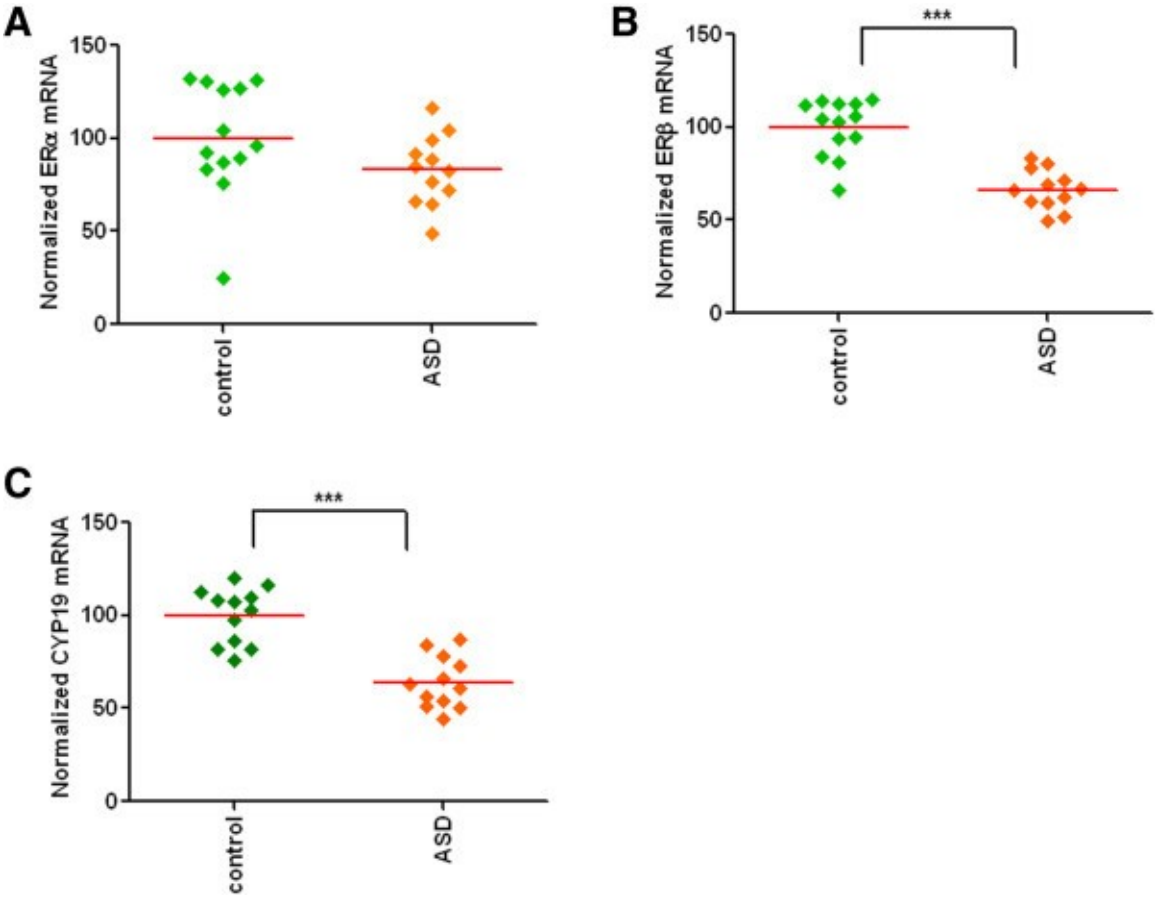

Figure 2. Decrease in mRNA levels of ERβ and CYP19A1, but no change in ERα mRNA in the middle frontal gyrus of ASD subjects.

(A) No significant change in ERα mRNA. (B) Significant reduction in ERβ mRNA in the ASD subjects. (C) CYP19A1 mRNA levels were significantly lower in the ASD subjects. mRNA levels were determined by qRT-PCR, and the values were normalized to the geometric mean of two control genes (GAPDH and β-actin). ***P <0.001 vs. controls.

Table 2. Correlations of mRNA transcripts with ADI-R scores

		Social interaction	Verbal communication	Non-verbal communication	Stereotyped behavior	Abnormality of development
ER co-repressors						
nCoR	r	−0.021	−0.202	0.479	−0.791	0.063
	P	0.961	0.745	0.277	**0.019**	0.893
	Adjusted *P*	1.000	1.000	1.000	0.095	1.000
SMRT	r	0.013	−0.090	−0.258	−0.489	−0.640
	P	0.976	0.866	0.576	0.219	0.122
	Adjusted *P*	1.000	1.000	1.000	0.876	0.610
ER co-activators						
CBP	r	−0.549	0.117	0.023	−0.261	−0.718
	P	0.126	0.825	0.956	0.498	**0.045**
	Adjusted *P*	0.504	1.000	1.000	1.000	0.225
P/CAF	r	−0.126	0.540	−0.319	0.515	−0.459
	P	0.747	0.268	0.441	0.156	0.253
	Adjusted *P*	1.000	1.000	1.000	0.780	1.000
TIF-2	R	−0.103	0.687	−0.217	0.536	−0.325
	P	0.792	0.132	0.606	0.137	0.432
	Adjusted *P*	1.000	0.660	1.000	0.660	1.000
SRC-1	r	−0.109	0.065	−0.876	0.294	−0.774
	P	0.798	0.917	**0.010**	0.480	**0.041**
	Adjusted *P*	1.000	1.000	**0.049**	1.000	0.164
AIB-1	r	0.274	0.200	0.099	−0.582	−0.000
	P	0.475	0.703	0.814	0.099	0.999
	Adjusted *P*	1.000	1.000	1.000	0.495	1.000
Estrogen receptor						
ERα	r	0.081	−0.210	0.265	−0.157	0.505
	P	0.848	0.735	0.566	0.710	0.248
	Adjusted *P*	1.000	1.000	1.000	1.000	1.000
ERβ	r	0.112	−0.203	0.192	−0.215	0.316
	P	0.791	0.743	0.680	0.610	0.489
	Adjusted *P*	1.000	1.000	1.000	1.000	1.000
Enzyme						
CYP19A1	r	0.405	−0.746	−0.143	−0.227	0.148
	P	0.320	0.148	0.760	0.589	0.751
	Adjusted *P*	0.740	1.000	1.000	1.000	1.000

ADI-R scores were available for only 9 of the 13 ASD subjects. Adjusted P denotes P values adjusted using the Holm-Bonferroni sequential correction for multiple comparisons; r, Pearson correlation; P, level of significance. Bolded text indicates significant P values.

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Decrease in mRNA levels of ER co-activators (SRC-1, CBP and P/CAF), but no change in ER co-repressors (SMRT and nCoR) in the middle frontal gyrus of ASD subjects

Postmortem interval, storage time, sample pH, and RNA integrity number were entered as covariates in the model to analyze SRC-1 mRNA data. None of the covariates in the model demonstrated a significant main effect when they were entered in the model with affection status. The predicted main effect of affection status on SRC-1 expression was statistically significant (Figure 2A) [$F(1, 18) = 11.05$, $P = 0.004$, $\eta 2p = 0.380$]. We found a significant reduction in SRC-1 mRNA (34% decrease) in the middle frontal gyrus of subjects with ASD (EMM = 63.33, SE = 6.26) as compared to controls (EMM = 95.85, SE = 6.26). The predicted main effect of affection status on CBP mRNA was statistically significant, when postmortem interval, storage time, sample pH, and RNA integrity number were entered as covariates in the model (Figure 2B) [$F(1, 18) = 53.15$, $P < 0.0001$, $\eta 2p = 0.747$]. None of the covariates in the model achieved statistical significance. Overall, subjects with ASD (EMM = 21.86, SE = 6.65) demonstrated much lower expression (77% decrease) of CBP than the control group (EMM = 95.64, SE = 6.33). Data on P/CAF mRNA was analyzed by considering postmortem interval, storage time, sample pH, and RNA integrity number as covariates in the model. However, none of these covariates remained significant predictors when they were entered into the model with affection status as an independent variable. The main effect of affection status on P/CAF expression was found significant (Figure 2C) [$F(1, 18) = 33.53$, $P < 0.0001$, $\eta 2p = 0.651$]. Moreover, a significant reduction in P/CAF mRNA expression (52% decrease) was found in the middle frontal gyrus of subjects with ASD (EMM = 47.47, SE = 5.71) as compared to controls (EMM = 97.80, SE = 5.44). Storage time, sample pH, and RNA

integrity number were included in the model as covariates to analyze the mRNA data on TIF-2. Among these covariates, storage time [$F(1, 20) = 4.70$, $P = 0.042$, $\eta 2p = 0.19$] and sample pH [$F(1, 20) = 4.32$, $P = 0.05$, $\eta 2p = 0.18$] remained significant covariates in the model. The predicted main effect of affection status on TIF-2 expression did not achieve statistical significance (Figure 2D) [$F(1, 20) = 1.49$, $P = 0.236$, $\eta 2p = 0.07$]. Similarly, no significant effect of affection status was observed on AIB-1 mRNA expression (Figure 2E) ([$F(1, 20) = 1.37$, $P = 0.264$, $\eta 2p = 0.06$].

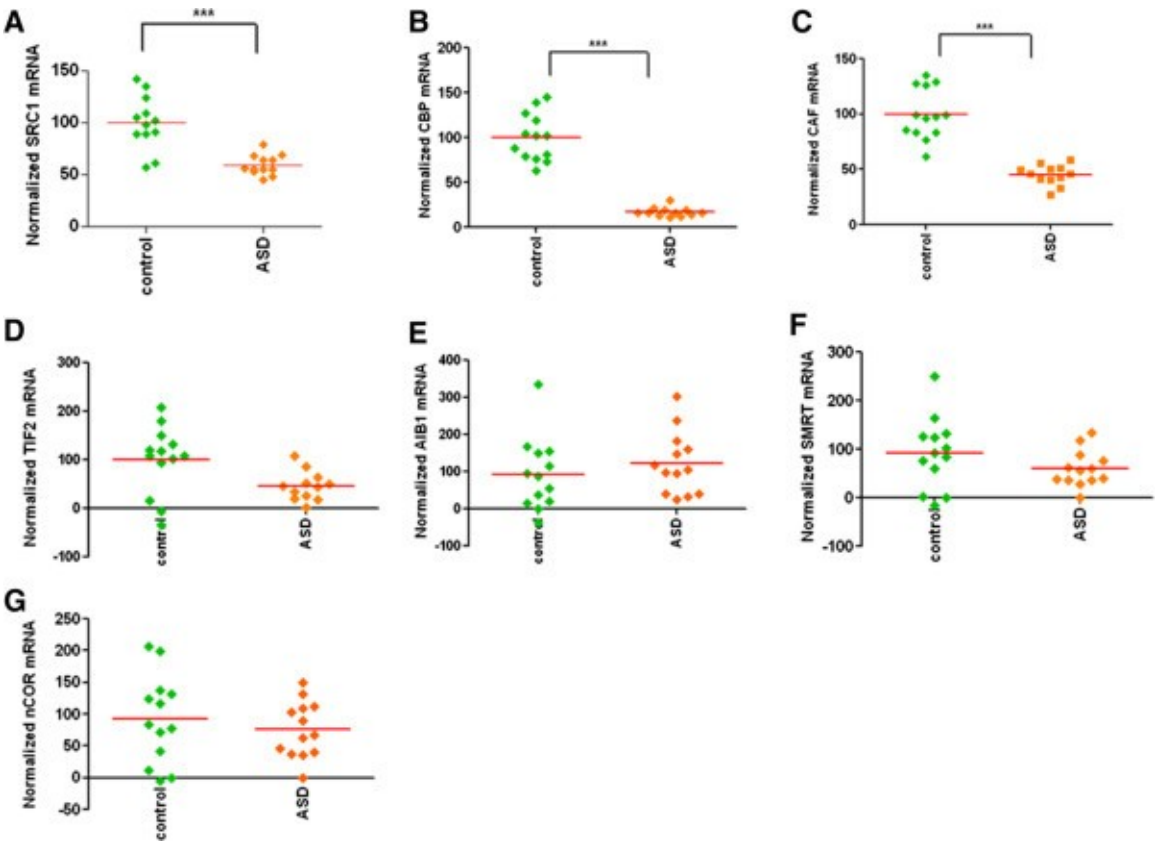

Figure 3. Decrease in mRNA levels of ER co-activators (SRC-1, CBP, and P/CAF), but no change in ER co-repressors (SMRT and nCoR) in the middle frontal gyrus of ASD subjects.

(A–E) Normalized mRNA expression of ER co-activators: (A) SRC-1, (B) CBP, (C) P/CAF, (D) TIF-2, and (E) AIB-1. (F and G) Normalized mRNA expression of ER co-repressors: (F) SMRT and (G) nCoR. mRNA levels were determined by qRT-PCR, and the values were normalized to the geometric mean of two control genes (GAPDH and β-actin). ***P <0.001 vs. controls.

Next, we examined whether the mRNA levels of ER co-repressors (SMRT and nCoR) are altered in the postmortem brain tissues of subjects with ASD. With postmortem interval and storage time entered as covariates in the model, the predicted main effect of affection status on SMRT expression was not statistically significant (Figure 2F) [$F(1, 17) = 0.013$, $P = 0.91$, $\eta 2p = 0.001$]. We found that storage time [$F(1, 17) = 7.51$, $P = 0.014$, $\eta 2p = 0.306$] remained a statistically significant covariate in this model. Data on nCoR mRNA was analyzed by considering postmortem interval, storage time, sample pH, and RNA integrity number as covariates in the model. No main effect of affection status was found on nCoR expression (Figure 2G) [$F(1, 17) = 0.046$, $P = 0.83$, $\eta 2p = 0.003$]. Storage time [$F(1, 17) = 9.59$, $P = 0.007$, $\eta 2p = 0.361$] was the only statistically significant covariate found in this model.

We observed some significant associations between the co-factors and the ADI-R scores in the subjects with ASD (Table 2). SRC-1 mRNA was negatively correlated with non-verbal communication ($r = -0.876$, $P = 0.01$) and abnormality of development ($r = -0.774$, $P = 0.041$) scores. A significant negative correlation was found between CBP mRNA and abnormality of development score ($r = -0.718$, $P = 0.045$). In addition, nCoR mRNA was negatively associated with stereotyped behavior score ($r = -0.791$, $P = 0.019$).

Correlations between mRNA expression of ERs, CYP19A1, and co-factors in the middle frontal gyrus of study subjects

We used Spearman's correlation to examine the relationships between mRNA expression of ERs, CYP19A1, and co-factors in the middle frontal gyrus of the study subjects.

Additional file 3: Table S3 depicts the correlations between the mRNA transcripts. ERα was positively correlated with ERβ and CYP19A1 mRNA levels. A large positive correlation was observed between ERβ and CYP19A1. In addition, ERβ was positively correlated with SRC--1, P/CAF, and CBP. Similarly, CYP19A1 was positively correlated with SRC1, P/CAF, and CBP. We also found significant correlations in the mRNA levels between the co-factors. Positive associations were found between SRC1 and P/CAF, CBP, TIF-2, SMRT, or nCoR. TIF-2 was positively correlated with P/CAF and CBP. In addition, we observed positive correlations between P/CAF and CBP mRNA levels, and CBP with SMRT expression. Interestingly, a significant positive correlation was also found in the mRNA levels between the co-repressors SMRT and nCOR. We did not find any significant correlation between AIB-1 and ERs, CYP19A1, or other co-factors examined in this study.

Decrease in ERβ and CYP19A1 protein levels in the middle frontal gyrus of ASD subjects

Given that we found significant reductions in ERβ and CYP19A1 mRNA levels in the middle frontal gyrus of subjects with ASD, we next determined the expression of the ERα, ERβ, and CYP19A1 at the protein level. We did not find any significant change in ERα protein levels in the middle frontal gyrus of subjects with ASD as compared to controls (Figure 3A). We found a significant reduction in ERβ protein (Figure 3B; $P < 0.05$) and CYP19A1 (Figure 3C; $P < 0.05$) levels in the middle frontal gyrus of subjects with ASD as compared to controls. Next, we examined whether the co-factors whose levels were altered in the ASD interact with ERβ in the brain samples. We performed immunoprecipitation assays to determine possible conjugation of P/CAF, SRC-1, and

CBP with ERβ in the middle frontal gyrus of control subjects, and each of these immunoprecipitates was examined for co-purification of ERβ by western blot. ERβ was detected in P/CAF, SRC-1, or CBP immunoprecipitates, but not in the control IgG (Additional file 4: Figure S1).

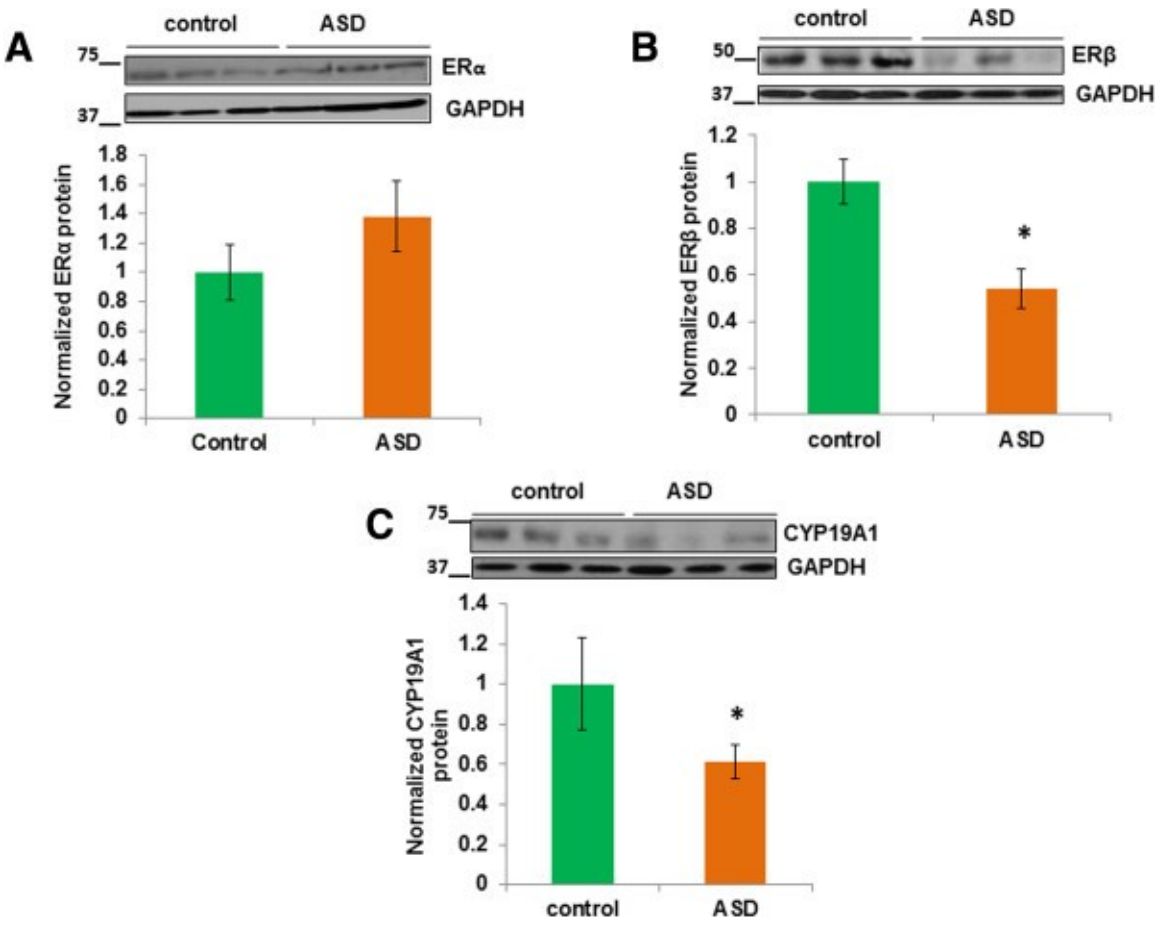

Figure 4. Decrease in ERβ and CYP19A1 protein levels in the middle frontal gyrus of ASD subjects. ERα, ERβ, and CYP19A1 protein levels were determined by western blot analysis.

The upper panels show representative autoradiogram of (A) ERα, (B) ERβ, or (C) CYP19A1 and GAPDH, and the lower panels represent fold-change in normalized ERα, ERβ, and CYP19A1 protein levels, respectively. Results are mean ± SEM vs. controls. *P <0.05 vs. controls.

Discussion

We have found, for the first time, alterations in ERβ transcriptional regulation in the brain of ASD subjects. We also report a significant decrease in CYP19A1 expression in ASD subjects. The above changes were associated with alterations in ER co-activators in the same study subjects. We did not find a significant difference in ERα expression between ASD and controls. Together, these findings suggest alterations in ER signaling in ASD.

The decrease in ERβ mRNA and protein expression in the middle frontal gyrus of ASD subjects found in this study is consistent with the increasing evidence for the role of estrogen signaling in the etiology of ASD. A significant association of the ERβ gene with autism traits as measured by the Autism Spectrum Quotient and the Empathy Quotient has been reported in ASD subjects (147). ERβ is abundantly expressed in the cortex (155). Moreover, ERβ plays an important role in neurodevelopment, and ERβ knockout, but not ERα knockout mice show defects of neuronal migration (156). It is known that ERα and ERβ have distinct tissue expression profiles, and have different cellular functions (19, 99, 158). ERα is involved in mediating estrogen action on reproductive organs and reproductive behavior, whereas ERβ is known to mediate some of the effects of estrogens on behaviors that are not specifically associated with reproduction, such as locomotor activity, fear responses, anxiety, and learning (148). ERβ knockdown has been shown to abolish E2-induced reductions in depressive behavior in mice (99, 157, 158). Moreover, administration of ERβ agonist or selective ligand has been shown to reduce anxiety-type behavior (158) and depressive behavior (159) in rats. Activation of ERβ with the specific agonist WAY-200070 in cortical neurons results in increased spine

density and PSD-95 (postsynaptic density-95) accumulation in membrane (160). Together, these results suggest that ERβ-mediated mechanism(s) are important for E2-induced neuronal plasticity.

The decrease in ERβ expression found in the ASD subjects might be the result of transcriptional regulation, either through methylation or by the regulation of genes of specific transcription factors binding to the ERβ promoter. Hypermethylation of the ERβ promoter is associated with a marked decrease in ERβ mRNA expression (161). It is known that estrogen binds to ER leading to a conformational change in ER. The estrogen-ER complex can bind directly to DNA via an estrogen responsive element or become attached to a transcription factor (19). It recruits a variety of co-regulators that result in the activation or repression of target genes by modifying chromatin structure. The p160/SRC (steroid receptor co-activator) family is one of the most studied classes of co-activators (164). Among the co-activators, SRC-1 and CBP exhibit autonomous histone acetyltransferase activity that promotes efficient transcription. In contrast, in the absence of ligands, ER associates with co-repressors nCoR or SMRT to mediate transcriptional repression of target genes through the histone deacetylase activity of the co-repressors (163-165). The present study revealed a novel finding that ERβ expression levels correlated with ER co-activators, SRC-1, P/CAF, and CBP. Moreover, we observed that the above co-factors interact with ERβ in human middle frontal gyrus. It is known that ERβ can antagonize ERα-dependent transcription in cells (166). In addition, ERβ and its variant, ERβ2, have been shown to increase the proteolytic degradation of ERα (167). Thus, it is possible that the changes in the expression levels of ERs and co-

factors observed in our study might influence the estrogen receptor signaling machinery and might play important roles in the pathophysiology of ASD.

Our findings demonstrate that CYP19A1 expression is significantly lower in the brain of ASD subjects. Earlier studies have reported the expression of CYP19A1, the key enzyme required for estrogen production, in the cortex (93). Furthermore, CYP19A1 is enriched at synapses and localizes to presynaptic structures in cortical neurons (160), suggesting that brain-synthesized estrogen plays an important role in neuronal function (125). The decrease in CYP19A1 could lead to reduced conversion of testosterone to estradiol resulting in increased levels of testosterone as observed in ASD subjects (168). Our data is in agreement with a previous finding on reduced aromatase protein levels in the frontal cortex of ASD subjects (9). An earlier genetic study has reported association between androgen receptor and ASD, suggesting an important role of androgen signaling in ASD (169). Further studies should examine the mRNA and protein levels of androgen receptors in the brain samples from ASD and control subjects, and such information would be helpful to better understand the relationship between estrogen-related and testosterone-related signaling pathways in ASD.

Conclusions

We have identified dysregulation of ERβ, CYP19A1, and co-activators associated with ER signaling in the middle frontal gyrus of ASD subjects with a significant association between these molecules. Our data suggest that a coordinated regulation of ER signaling molecules plays an important role in ER signaling in the brain, and that this network may

be impaired in subjects with ASD. Although we found a large, significant association between the co-factor mRNA transcripts (SRC-1, CBP, and nCoR) and ADI-R scores in ASD subjects, its implication is unclear. Moreover, the robust reduction found in CBP mRNA expression in ASD subjects needs further investigation. CBP is known to be associated with other steroid receptors, including progesterone receptor, thyroid hormone receptors, and retinoid receptors (161). It is important to examine whether the complex formation of ERβ with CBP is indispensible for ER-dependent neuronal plasticity and ASD-like behavior. Moreover, the present data was collected in a relatively smaller number of study subjects, which needs further investigation using large samples before a conclusion can be drawn. Since brain tissue from individuals with ASD is quite scarce, lymphoblastoid cell lines that are banked for ASD cohorts (though there are several limitations including difference in tissue type and difficulties in transformation procedure) could provide a large sample of biological material to understand the pathophysiology of ASD. Future studies will investigate the mechanism of regulation of ERβ in ASD, which might lead to a better understanding of the pathophysiology and provide new avenues of treatment of this disorder.

2. Estrogen receptor β attenuates endoplasmic reticulum stress---induced autism spectrum disorder---like behavior through IRE---1/XBP1 pathway

Abstract

Impaired social interaction is a key feature of several major psychiatric disorders including depression, autism and schizophrenia. However, little is known about the cellular mechanisms regulating adult social interaction. Endoplasmic reticulum (ER) plays key roles in protein modification, folding, and maturation of proteins. Although ER stress has been implicated in the above neuropsychiatric disorders, the role of ER stress in social behavior is not known. In this study, treatment with tunicamycin, an ER stress inducer enhanced the phosphorylation level of inositol-requiring ER-to-nucleus signal kinase 1 (IRE1) and increased X-box-binding protein 1 (XBP1) mRNA splicing activity in the mouse prefrontal cortex (PFC), whereas inhibition of IRE1/XBP1 pathway by either a viral particle or pharmacological approach attenuated social behavioral deficits caused by tunicamycin treatment. Reduced estrogen receptor beta (ERβ) protein levels were found in PFC of male mice following tunicamycin treatment. Pretreatment with an ERβ specific agonist, ERB-041 significantly attenuated tunicamycin-induced deficits in social behavior, and activation of IRE1/XBP1 pathway in mouse PFC. Also, ERB-041 inhibited tunicamycin-induced increases in functional connectivity between medial prefrontal cortex (mPFC) and dorsal or ventral hippocampus in male mice. Together, these results show that ERβ attenuates ER stress-induced deficits in social behavior through IRE-1/XBP1 pathway.

Introduction

Impaired social interaction is a key feature of several major psychiatric disorders including depression, autism and schizophrenia (American Psychiatric Association, 2013). Previous studies have shown the role of various genetic as well as epigenetic factors in social behaviors in both nonhuman primates and rodents (170). In addition, postmortem studies have linked the changes in the expression of various genes involved in synaptic plasticity to social behavior in the above psychiatric conditions (171-174). Furthermore, functional magnetic resonance imaging (fMRI) studies have shown altered functional connectivity between cortical regions across a variety of diseases with social behavior deficits, including autism (175, 176), schizophrenia (177), and major depression (178). The above findings are further supported by in vivo electrophysiology studies in rodents showing the role of abnormal brain connectivity in social behavior deficits (179). However, little is known about the cellular mechanisms regulating adult social interaction.

The ER is an intracellular organelle that serves key functions involving in the biosynthesis of membrane and secretory proteins, synthesis of lipids, and maintenance of intracellular calcium homeostasis (180). A number of pathophysiological or environmental stimuli can impose stress on the ER and subsequently interrupt the protein folding process in the ER, leading to accumulation of unfolded or misfolded proteins in the ER lumen called ER stress (129). Three ER-resident proteins have been identified as sensors of ER stress: IRE1 (inositol-requiring protein 1), PERK [PKR (double-stranded-RNA-dependent protein kinase)-like ER kinase] and ATF6 (activating transcription

factor (128; 113). IRE1 is a type 1 transmembrane serine/threonine receptor protein kinase which functions as a sensor for misfolded/unfolded proteins in the ER lumen. Activated IRE1 induces the splicing of *XBP1* (X-box-binding protein 1) mRNA by cleaving off its intron (181; 182). PERK is a type 1 transmembrane protein kinase that transmits stress signals in response to the perturbation of protein folding (183). When activated, PERK phosphorylates the α subunit of eIF2 (eukaryotic initiation factor 2) leading to the translation of ATF4 and activation of the CHOP promoter (183, 184). ER stress activates ATF6 by translocating it from the ER to Golgi complex, where it is cleaved by the Golgi-resident serine proteases S1P and S2P (site 1 and site 2 proteases respectively) (185, 186) resulting in the activation of the transcription of UPR targets such as GRP78, CHOP and XBP1 (182; 186). The UPR is generally a pro-survival mechanism, mediated by translation arrest and the induction of a number of transcription factors and chaperone proteins that function to restore the restore ER homeostasis and help the cells adapt to ER stress conditions. However, when ER stress is prolonged or the degree of ER stress is too severe, UPR signaling can initiate programmed cell death by activating stress-induced pro-apoptotic factors (130, 131). Numerous studies have demonstrated that ER stress is involved in autism (7, 187, 127, 128), depression (188) and schizophrenia (189, 190). However, it remains unclear whether elevated ER stress leads to impaired social interaction.

To investigate the role of ER stress in social behavior, we induced ER stress in mice by tunicamycin administration. Tunicamycin is an inhibitor of the UDP-N-acetylglucosamine-dolichol phosphate N-acetylglucosamine-1-phosphate transferase

(GPT), therefore blocking the initial step of glycoprotein biosynthesis in the ER leading to ER stress. We found that tunicamycin treatment induces social interaction deficits and alterations in functional brain connectivity in male mice. We observed significant reduction in ERβ protein levels in the PFC of male mice following tunicamycin treatment, and pretreatment with ERβ agonist, ERB-041 could attenuate tunicamycin treatment–induced social interaction deficits and changes in brain connectivity. Indeed, pretreatment with ERB-041 significantly decreased the ER stress-induced increase in IRE1 activation in mouse PFC suggesting that inhibition of IRE1/XBP1 signaling could a potential mechanism involved in ERβ signaling mediated rescue of impaired social behavior.

Methods

Animals

Adult (8-10 week old) C57BL/6J male and female mice were purchased from Charles River Laboratories (Wilmington, MA, USA). Mice were housed in groups of 4 mice in standard polypropylene cages in 12-h light-dark cycle. All behavior experiments were performed at 8-10 weeks of age. Separate cohorts of animals were used for different behavioral analyses due to the acute response of tunicamycin and the length of time needed to perform behavioral testing. The same animals that were used for behavioral analysis were used for molecular studies. All experiments were in compliance with the US National Institute of Health guidelines and approved by Augusta University animal welfare guidelines.

Drug treatment

Mice were injected intraperitoneally with 1mg/kg tunicamycin (catalog #T7765; Sigma, St. Louis, Missouri) dissolved in DMSO (vehicle control). This dose was selected based on previous studies showing this dose to actively induce ER stress in mice (131). The selective ERβ-specific agonist, ERB-041 (catalog #PZ0183; Sigma, St. Louis, Missouri; 1 mg/kg) or IRE1 inhibitor (4μ8c; catalog #4479; R&D Systems; Minneapolis, Minnesota; 10 mg/kg) was administered 30 minutes before tunicamycin injection (191, 192). Mice were monitored for adverse effects of treatments. Behavioral experiments were performed 12 hours after tunicamycin injection.

Surgery and EEG recordings

Anesthesia was induced with ketamine-xylazine (80mg/kg-5mg/kg) in sterile distilled water. The mouse was then placed in a stereotaxic frame with heating pad (Kopf, Tujunga, California). The skull was exposed and PFA-coated tungsten wire electrodes were placed into bur holes in mPFC (1.8mm anterior, 0.5mm lateral, 1.5mm deep), dorsal hippocampus (1.9mm posterior, 1.4mm lateral, 1.35mm deep), and ventral hippocampus (3.2mm posterior, 3.1mm lateral, 3.85mm deep) relative to bregma. Reference and ground screws attached to electrodes were placed at neutral locations superficial to parietal and occipital cortex, respectively. Electrodes were soldered to a 7 pin adaptor and fixed to the skull with dental cement (Co-oral-lte Dental Manufacturing Company, Diamond Springs, California). Once the cement had dried, animals were removed from the stereotaxic instrument, injected intraperitoneally with 0.1 mL saline and buprenorphine (0.2 mg/kg) and returned to a clean cage and singly housed. Mice were

given an additional buprenorphine injection (0.2 mg/kg) the following day. Mice were monitored daily for 2 weeks to assure proper recovery. Experiments were performed 2 weeks post-surgery. Recording quality was tested and animals with poor signals were excluded from the study.

EEG recordings were performed 12 hours after vehicle (DMSO, i.p.), tunicamycin (1mg/kg, i.p.), or ERB-041 (1mg/kg, i.p.) + tunicamycin (1mg/kg i.p.), between 9am and 12pm. For recording purposes wireless Neurologger 2a (Evolocus LLC, Tarrytown, NY) was used to monitor EEG activity. The Neurologger 2a apparatus was attached to the mounted 7 pin adaptor of the animal and allowed recording from up to 4 channels at a sampling rate of 1600Hz (reference assignment 2:2; oversampling x4). Recordings were downloaded offline to a PC for analysis. Data were analyzed in MATLAB (MathWorks, Natick, Massachusetts; RRID:SCR_001622) using custom scripts.

Stereotaxic Injection of Lentivirus

IRE1 shRNA (m) Lentiviral (LV) Particles and its control shRNA LV particles were purchased (Santa Cruz, CA, USA). LV-IRE1-shRNA is a pool of concentrated, transduction-ready viral particles containing 4 target-specific constructs that encode 19-25 nt (plus hairpin) shRNA designed to knock down gene expression. shRNA lentiviral particles frozen stock contains a concentration of 1.0×10^6 infectious units of virus in Dulbecco's Modified Eagle's Medium with 25 mM HEPES pH 7.3. Lentiviral particles were infused into mouse PFC by stereotaxic microinjection (coordinates: x .5 mm (lateral), y 1.0 mm (anteriorposterior A–P), with respect to bregma at 0), z 1.0 mm

(dorso−ventral D–V with brain surface at 0). at a rate of 0.2 µl/min at each site (Stoelting Co) (193).

Behavioral experiments

Behavioral testing was performed in a room with constant background sound and ambient lighting approximately 25-30 Lux (lumen/m2) unless noted. Temperature and pressure in behavioral rooms are monitored and kept constant. Animals are transferred in their home cages to behavioral rooms at least 1 hour before testing and allowed to habituate to the testing room. All behavioral experiments were scored blind to treatment.

Three Chamber Test

This test was performed to measure sociability and social deficits. The test mouse was placed in a box with 3 chambers. Each chamber is 19 cm x 45 cm x 22cm and the dividing walls are made from clear Plexiglas®, with openings on each wall for free access to the other two chambers. Two identical wire containers that were large enough to house a single mouse were placed vertically inside the apparatus with one in each side chamber and weighted down. The test mouse was habituated to the apparatus for 5 minutes while freely exploring. After the habituation period, the stranger mouse was placed in one of the wire containers while the test mouse was still allowed to freely move outside of the container. The wire containers allow air exchange between the interior and exterior, but the holes are small enough to prevent direct physical contact between the stranger mouse and test mouse. The free test mouse was allowed to interact through the wire container with the stranger mouse for 5 minutes. During this time, time spent in

chambers (stranger mouse, empty cage, and center) was recorded by an examiner with a stopwatch. The stranger mouse chamber is defined as the chamber containing the wire container with the stranger mouse inside. The empty cage chamber is the chamber containing an empty wire container. The stranger mouse was a mouse of similar age, same sex, and similar weight as the test mouse. The examiner was blinded to the treatment groups and sex.

Reciprocal Social Interaction Test

This test was performed to measure sociability and social deficits. The test mouse was placed in a neutral box (57cm x 45cm x 22cm) made from clear Plexiglas® and allowed to habituate for 5 minutes. After habituation, a stranger mouse was placed in the box and the test mouse was allowed to freely interact with the stranger mouse. Interaction is defined as close physical contact, nose to nose sniffing, anogenital sniffing, and grooming. Time spent interacting (initiated by the test mouse) was recorded by an examiner with a stopwatch. The stranger mouse was a mouse of similar age, same sex, and similar weight as the test mouse. The examiner was blinded to the treatment groups and sex.

Open field test

Mice were placed in an open field chamber and total distance traveled (cm) was measured over a 15 minute period**.** The open field chamber was (40 × 40 × 40 cm) and made of white opaque Plexiglas®. A video camera was fixed over the chamber by an adjacent rod. Ethovision XT 10 (Noldus Information Technologies Inc, USA) software

was used for analysis. Trial totals for all parameters were taken. The open field test was performed as a measure of locomotor activity.

Western blotting

Animals were sacrificed by cervical dislocation after being anesthetized using isoflurane. PFC tissue was homogenized in a tissue lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1.0% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 2 mM EDTA, 6 µM PMSF, and 1.0% Triton X-100 supplemented with protease inhibitor cocktail (Sigma, St. Louis, Missouri). The homogenate was centrifuged at 13,000 rpm for 10 min at 4°C and the supernatant was used for protein estimation by the bicinchoninic acid method (BCA Protein Assay Kit, Sigma, St. Louis, Missouri). Samples (30 µg) were subjected to SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was blocked for 1 hour in PBS with Tween 20 and 5%-10% non-fat milk followed by overnight incubation with a primary antibody. Blots were incubated in the appropriate primary antibody specific for IRE1 (Santa Cruz Biotechnology, Dallas, TX, catalog #SC-20790, 1:200 and Cell Signaling, Boston, MA, catalog #3294, 1:500, RRID:AB_2098712), phosphoIRE1 (Novus Biologicals, Littleton, CO, catalog#NB100-2323, 1:1000, RRID:AB_10145203), tubulin (Cell Signaling, Boston, MA, catalog#2144, 1:10,000), ERβ (Abcam, Cambridge, UK; 3576; 1:1,000); ERα (Santa Cruz Biotechnology, Dallas, Texas; 71064; 1:1,000, RRID:AB_1122667); and developed with the SuperSignal West Pico Chemiluminescent substrate system (Thermo Fisher Scientific, West Columbia, SC). Optical densities of the bands were analyzed using ImageJ software (NIH, RRID:SCR_003070). For analysis, protein levels were

normalized to tubulin levels, and then expressed as a fold change of that in control animals. For figure panels, contrasts have been adjusted linearly for easier viewing of bands.

Quantitative reverse transcriptase PCR (qRT-PCR)

RNA was purified using a commercially available kit (SV RNA Isolation, Promega, Madison, WI, USA), qRT-PCR was performed on a MasterCycler (Eppendorf, Hamburg, Germany) using a SuperScript III Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen, Carlsbad, CA, USA). Gene-specific primers were synthesized by Integrated DNA Technologies. Primers used are: CHOP-FP: 5'- CATACACCACCACACCTG AAAG -3', CHOP-RP: 5'- CCGTTTCCTAGTTCTTCC TTGC -3', sXBP1-FP: 5'- CTGAGTCCGAATCAGGTGCAG-3′, sXBP1-RP: 5'- GTCCATGGGAAGATGTTCTGG-3', RPS3-FP: 5'- *AATGAACCGAAGCACACCATA*-3', and RPS3-RP: 5'- *ATCAGAGAGTTGACCGCAGTT*-3'. Ct values of genes of interest were normalized to that of housekeeping genes (18S or RPS3).

Statistics

No statistical methods were used to predetermine sample sizes in mouse studies, but our sample sizes are similar to those reported in previous publications (193, 194, 195). For mouse studies, data were analyzed using two-tailed Student's t-tests (for two-group comparisons) or one-way or two-way Analysis of Variance (ANOVA; for multiple-group comparisons). $p < 0.05$ was considered significant. Post hoc analyses were carried out

using Bonferroni's test. All analyses were performed using SPSS Statistics 20 software (IBM).

Results

IRE-1/XBP-1 pathway mediates ER stress-induced social interaction deficits in mice. The IRE-1/XBP-1 pathway is the most conserved ER stress-response pathway. The activation of IRE-1 results in non-conventional splicing of the mRNA encoding the transcription factor X-box binding protein 1 (XBP1), generating a spliced active form of XBP1 (sXBP1) to initiate a major UPR program. To determine whether IRE-1/XBP-1 pathway plays any role in ER stress-induced ASD-like behavior, we first examined the phosphorylation status of IRE-1 in the prefrontal cortex (PFC, a key brain region mediating social cognition in both humans and rodents (183) of mice treated with tunicamycin (Figure 4a). We found significant increase in phosphoIRE1 levels in the PFC of tunicamycin-treated mice indicating activation of IRE-1 following ER stress (Figure 4b). Also, we found significant increase in splicing of XBP1 as determined by the mRNA levels of sXBP1 in PFC of tunicamycin-treated mice (Figure 4c).

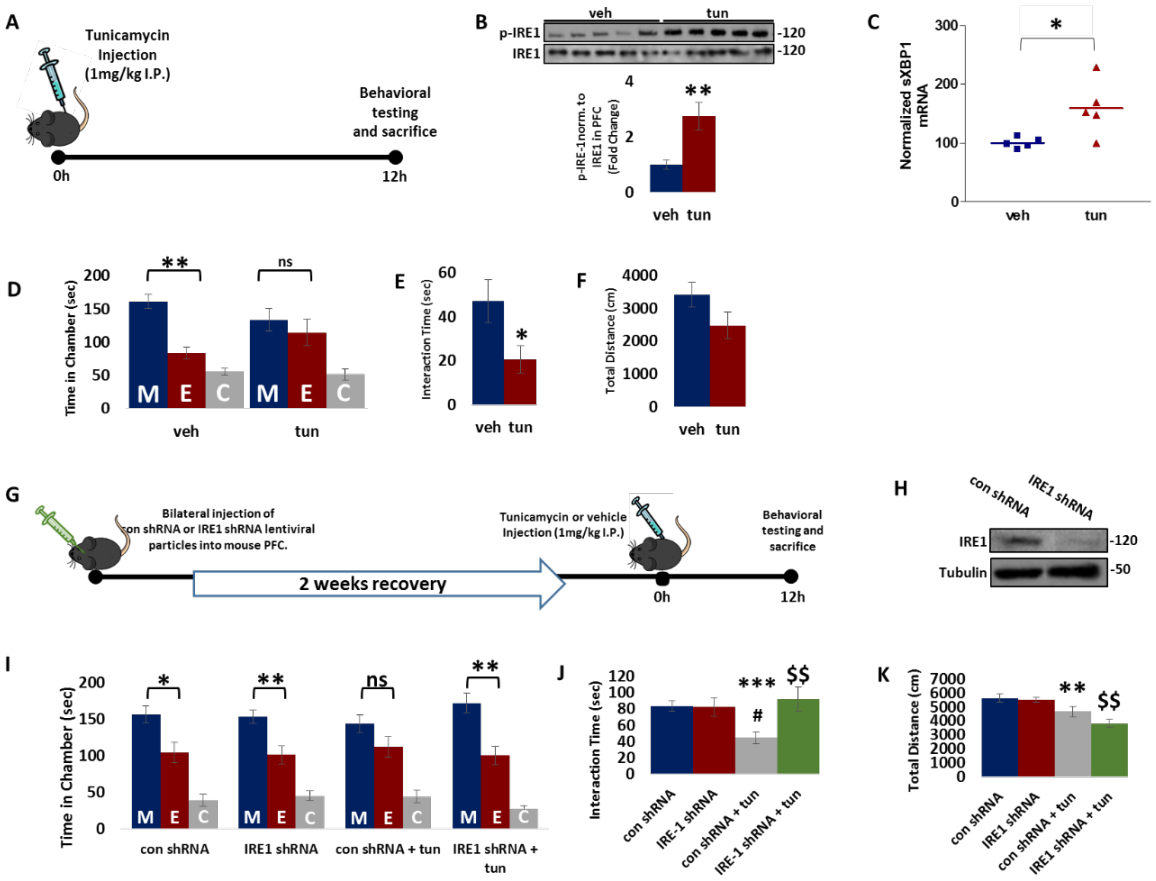

Figure 5. IRE-1 mediates ER stress-induced deficits in social behavior in male mice.

A) Treatment paradigm. Young adult male mice were injected intraperitoneally with tunicamycin (1mg/kg in DMSO) or vehicle (DMSO). Behavior tests were performed 12 h after tunicamycin injection. B) phospho-IRE1 and IRE1 protein levels were determined in the mouse PFC 12 h after tunicamycin injection. Top. Representative blot. Bottom. Quantification of phospho-IRE1 to IRE1 ratio. Protein levels were measured by western blot analysis. (n=6-8). C) mRNA levels of spliced XBP1 (sXBP1) were determined by qRT-PCR in the mouse PFC 12 h after tunicamycin injection. The Ct values were normalized to RPS3. (n=5). D) Three chamber test (n=10-13). E) Reciprocal social interaction test (n=10-13). F) Open field test. (n=8-9). G) Schematic representation of stereotaxic injection of control or IRE1 shRNA lentiviral particles into mouse PFC followed by tunicamycin treatment for 12 h. H) Representative immunoblot data showing IRE1 and tubulin expression in the PFC of mice injected with control or IRE1 shRNA particles. I) Three chamber test (n=10-13). J) Reciprocal social interaction test (n=10-13). K) Open field test. (n=8-9). Data are expressed as mean s.e.m. *p<0.05, **p<0.01 and ***p<0.001 vs. veh or con shRNA group. $p<0.05 and $$p<0.01 vs. tunicamycin-treated con shRNA group. #p<0.05 and ##p<0.01 vs. stranger mouse chamber. ns-non-significant. Student's t test or Two-way ANOVA.

To determine the effects of ER stress on social behavior, we performed three chamber test and reciprocal interaction test in mice treated with tunicamycin or vehicle. In Three-chamber test, we found that whereas vehicle-injected mice spent more time in the chamber housing stranger mouse than the empty cage chamber, tunicamycin-injected mice had no preference for either chamber ($p < 0.05$; Figure 4d). In Reciprocal Social Interaction test, tunicamycin-injected mice showed decreased interaction with a stranger mouse when compared with those from vehicle-treated group ($p < 0.05$; Figure 4e). These behavioral changes were not due to differences in mobility (open field test) between the two groups (Figure 4f).

To determine the direct role of IRE-1 in ER stress-induced social interaction deficits in male mice, we silenced IRE-1 expression in mouse PFC using lentiviral vectors expressing IRE-1 shRNA (Figure 4g). A significant reduction in IRE-1 protein levels was found in mouse PFC following IRE-1 shRNA administration ((Figure 4h). We found that tunicamycin induced deficits in three chamber test (Figure 4i) and reciprocal interaction test (Figure 4j) in control shRNA-treated mice, but not in IRE-1 shRNA-injected mice. Locomotor activity deficits in the tunicamycin treated groups is likely due to the combination of surgical and pharmacological insults experienced by these mice. There was no significant change between control shRNA + tunicamycin treated mice and IRE1 shRNA + tunicamycin treated mice, suggesting that the deficits are due to the combination of insults, not due to the lentiviral particle type (Figure 4k). The role of IRE-1 pathway in mediating ER stress-induced social behavior deficits was further confirmed using 4μ8C, which is known to potently inhibit *XBP1* mRNA splicing by IRE-1 (Fig. 5a).

4μ8C pre-treatment significantly attenuated the tunicamycin-induced changes in three chamber test (Fig. 5b) and reciprocal interaction test (Fig. 5c). We did not find any significant change in locomotor activity between groups (Fig. 5d).

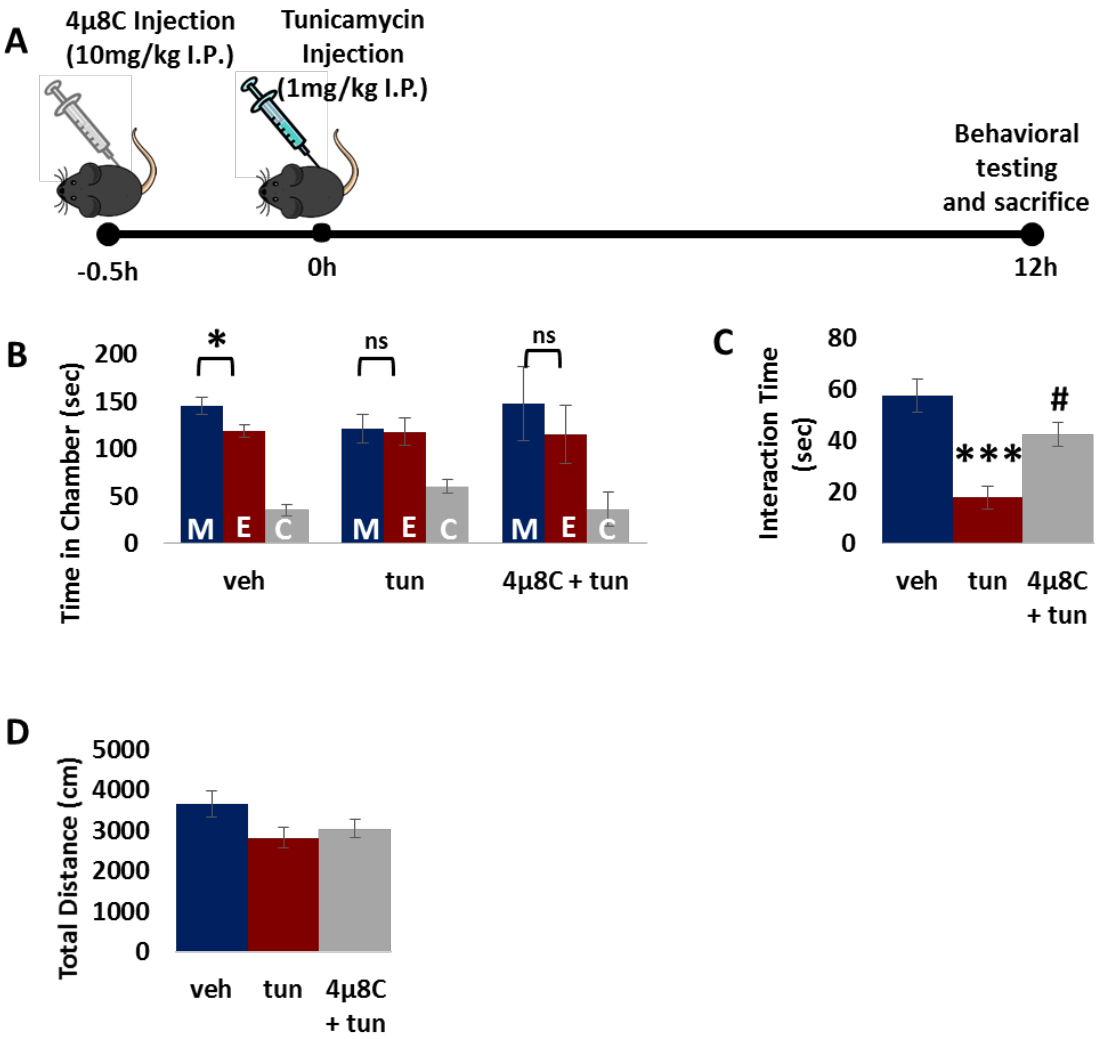

Figure 6. IRE-1 inhibition attenuates ER stress-induced social behavior deficits in mice.

Adult male mice were injected intraperitoneally with tunicamycin (1mg/kg in DMSO), vehicle (DMSO), or 4µ8C (10mg/kg in DMSO, 30 minutes before tunicamycin injection) and tunicamycin (1mg/kg in DMSO). A) Treatment paradigm. B) Three chamber test (*n*=10-13). C) Reciprocal social interaction test (*n*=10-13). D) Open field test. (*n*=8-9). Data are expressed as mean ±s.e.m. *p<0.05 and ***p<0.001 vs. vehicle. $^{\$}$p<0.05, $^{\$\$}$p<0.01, and $^{\$\$\$}$p<0.001 vs. tunicamycin. $^{\#}$p<0.05 vs. stranger mouse chamber.. ns=non-significant. One-way ANOVA. Two-way ANOVA for time in chamber (B).

Tunicamycin treatment does not induce social behavior deficits in female mice.

Next, we examined the effects of tunicamycin treatment on social interaction in female mice (Figure 6a). Tunicamycin treatment did not result in any significant changes in three chamber test (Figure 6b) and reciprocal interaction test (Figure 6c) in female mice.

Tunicamycin treatment induces decrease in ERβ protein levels in mouse PFC.

In order to examine whether tunicamycin treatment altered the expression of ERα and ERβ, we performed western blot analyses on PFC samples from vehicle and tunicamycin treated male mice. ERβ total protein levels were found to be significantly decreased in PFC ($p < 0.05$; Figure 7a) of mice treated with tunicamycin as compared to vehicle treated mice. No change in ERα protein levels was found in PFC following tunicamycin treatment (Figure 7b).

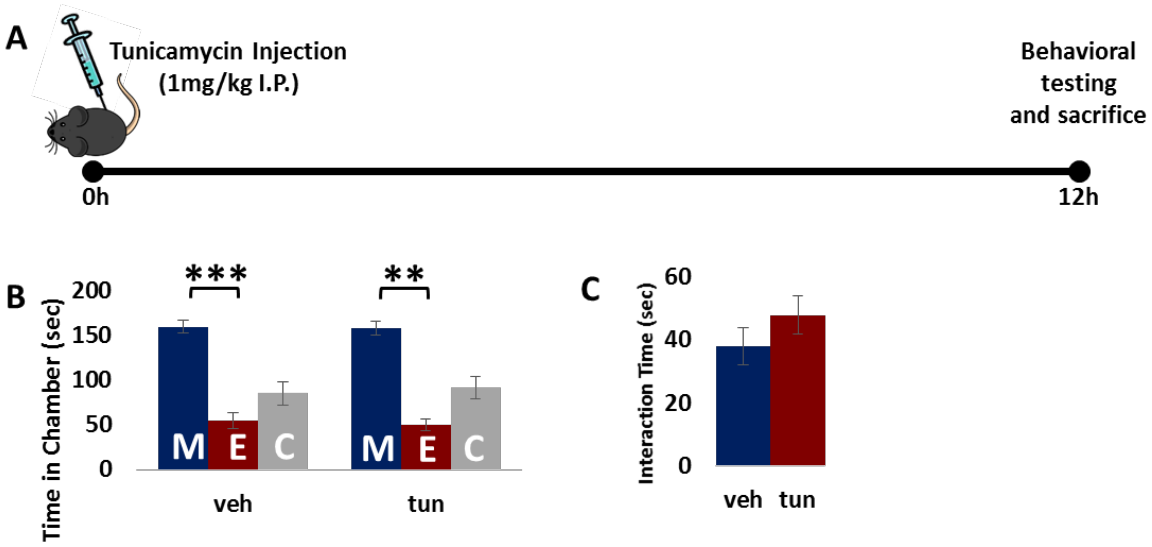

Figure 7. ER stress by tunicamycin treatment does not induce social behavior deficits in female mice.

Young adult female mice were injected intraperitoneally with tunicamycin (1mg/kg in DMSO) or vehicle (DMSO) and behavioral testing was performed 12 hours after injection. A) Treatment paradigm. B) Three chamber test (n=10-13). C) Reciprocal social interaction test Data are expressed as mean ±s.e.m. $^{**}p<0.001$ vs. stranger mouse chamber. Two-way ANOVA.

ERβ agonist ERB-041 attenuates ER stress-induced social interaction deficits in male mice.

If a reduction in ERβ signaling mediates the tunicamycin-induced deficits in social behavior in mice, activation of ERβ should ameliorate the behavioral signs. ERB-041 (2-[3-fluoro-4-hydroxyphenyl]-7-vinyl-1,3-benzoxazol-5-ol) is a selective ERβ agonist. Adult male mice were treated with ERB-041 30 min prior to tunicamycin injection and behavioral tests were performed 12 h later (Figure 7c). We examined the effects of ERB-041 on IRE-1/XBP1 pathway in PFC samples from tunicamycin-treated mice. We found that ERB-041 pretreatment significantly attenuated tunicamycin-induced increase in IRE-1 activation in PFC of tunicamycin-treated mice ($p < 0.05$; Figure 7d). In the behavioral tests, the tunicamycin-induced deficits in social behavior in three chamber test (Figure 7e) and reciprocal interaction test (Figure 7f) were significantly reduced by ERB-041 pre-treatment. No significant change in total distance traveled in open field test was found between the treatment groups (Figure 7g). These results strongly suggest that reduced ERβ signaling is involved in the social interaction deficits observed in tunicamycin-treated mice.

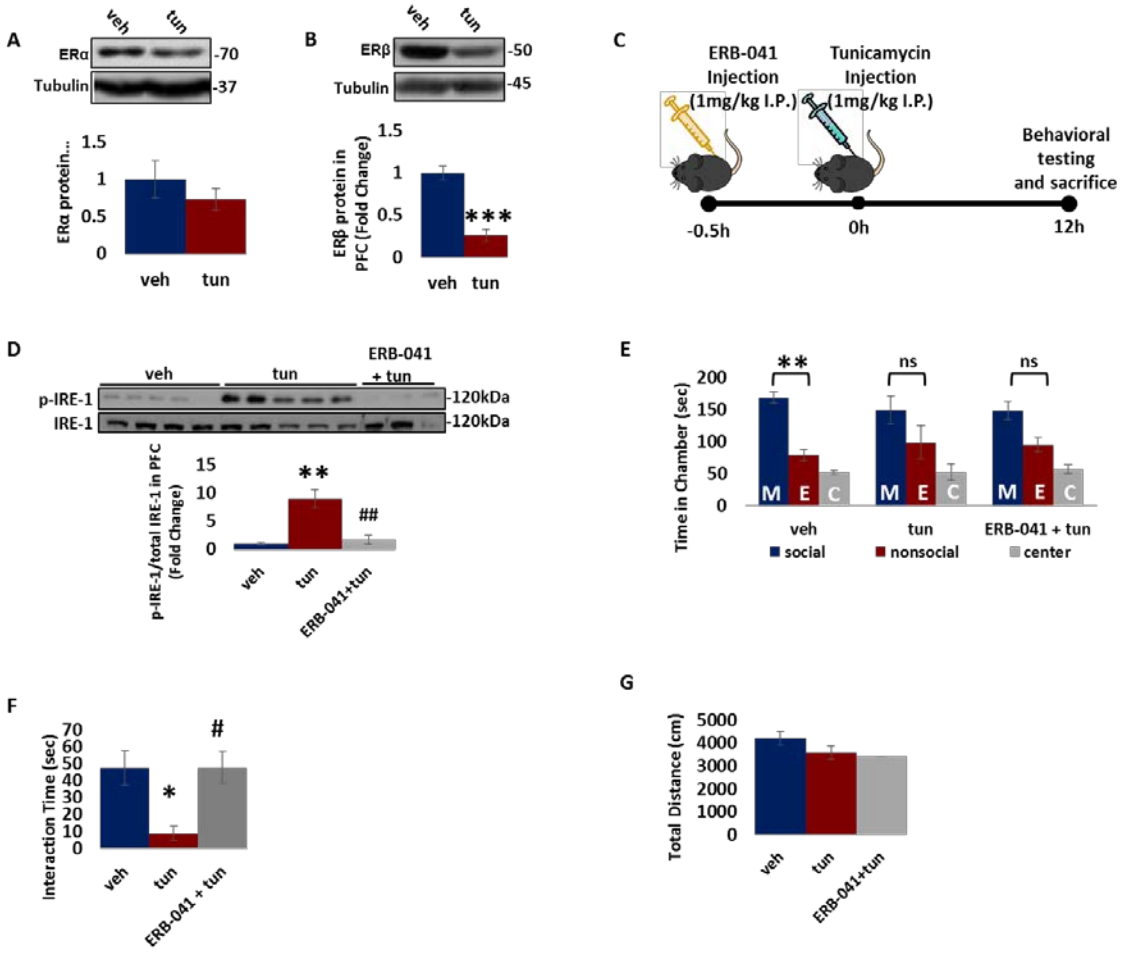

Figure 8. ERβ agonist ERB-041 attenuates ER stress-induced social behavior deficits in male mice.

Adult male mice were injected intraperitoneally with tunicamycin (1mg/kg in DMSO) or vehicle (DMSO). (A) ERβ and (B) ERα protein levels were determined in mouse PFC 12 h after tunicamycin injection. *Top.* Representative blot. *Bottom.* Quantification of ERβ or ERα protein. Protein levels were measured by western blot analysis and normalized to tubulin. Data are expressed as mean ±s.e.m. (*n*=5-8). *p<0.05 vs. vehicle; Student's *t* test. C) Treatment paradigm. Adult male mice were injected intraperitoneally with tunicamycin (1mg/kg in DMSO), vehicle (DMSO), or ERB-041 (1mg/kg in DMSO, 30 minutes before tunicamycin injection) and tunicamycin (1mg/kg in DMSO). Behavior was performed 12 hours after tunicamycin injection. D) phospho-IRE1 and IRE1 protein levels were determined in the mouse PFC 12 h after tunicamycin injection. *Top.* Representative blot. *Bottom.* Quantification of phospho-IRE1 to IRE1 ratio. Protein levels were measured by western blot analysis. (*n*=4-5). E) Three chamber test (*n*=10-13). F) Reciprocal social interaction test (*n*=10-13). G) Open field test. (*n*=8-9). Data are expressed as mean ±s.e.m. *p<0.05, **p<0.01 and ***p<0.001 vs. vehicle. $^{\$}$p<0.05 and $^{\$\$}$p<0.01 vs. tunicamycin. $^{\#\#}$p<0.01 vs. stranger mouse chamber. ns-non-significant. One-way ANOVA. Two-way ANOVA for time in chamber (G).

ERβ agonist ERB-041 attenuates ER stress-induced PFC-hippocampus hyperconnectivity in male mice.

Social behavior deficits are known to be associated with alterations in brain functional connectivity and our data impaired social behavior in ER stress-induced mice, so we examined whether ER stress induces changes in brain functional connectivity. The prefrontal cortex and hippocampus are key brain regions mediating social cognition in both humans and rodents (196), which is abnormal in ASD. Male mice (3 months old) were implanted with depth electrodes, and local field potentials (LFPs) were recorded with a wireless Neurologger 2A device mounted on the head of the animal (Figure 8a).

To quantify long-range functional connectivity, we measured the coherence of LFPs between the medial PFC (mPFC) and dorsal hippocampus (dHIPP) in mice treated with tunicamycin or vehicle. Coherence spectra indicated a significant increase in functional connectivity between mPFC and dHIPP in the theta (4–12 Hz), beta (15–25 Hz) and gamma (26–70 Hz) bands ($p < 0.05$; Figure 8b-c). Interestingly, ERB-041 pretreatment could significantly attenuate tunicamycin-induced increase in mPFC-dHIPP coherence ($p < 0.05$; Figure 8b-c). Also, we examined whether tunicamycin alters brain connectivity between mPFC and ventral HIPP (vHIPP) in mice. We found a decrease in coherence in theta band, but increase in gamma band between mPFC-vHIPP in mice treated with tunicamycin (Figure 8d-e). No significant change was found in mPFC-vHIPP beta band between vehicle and tunicamycin-treated mice. Moreover, the ERB-041 pretreatment significantly reduced the coherence in beta and gamma bands in tunicamycin-treated mice (Figure 8d-e).

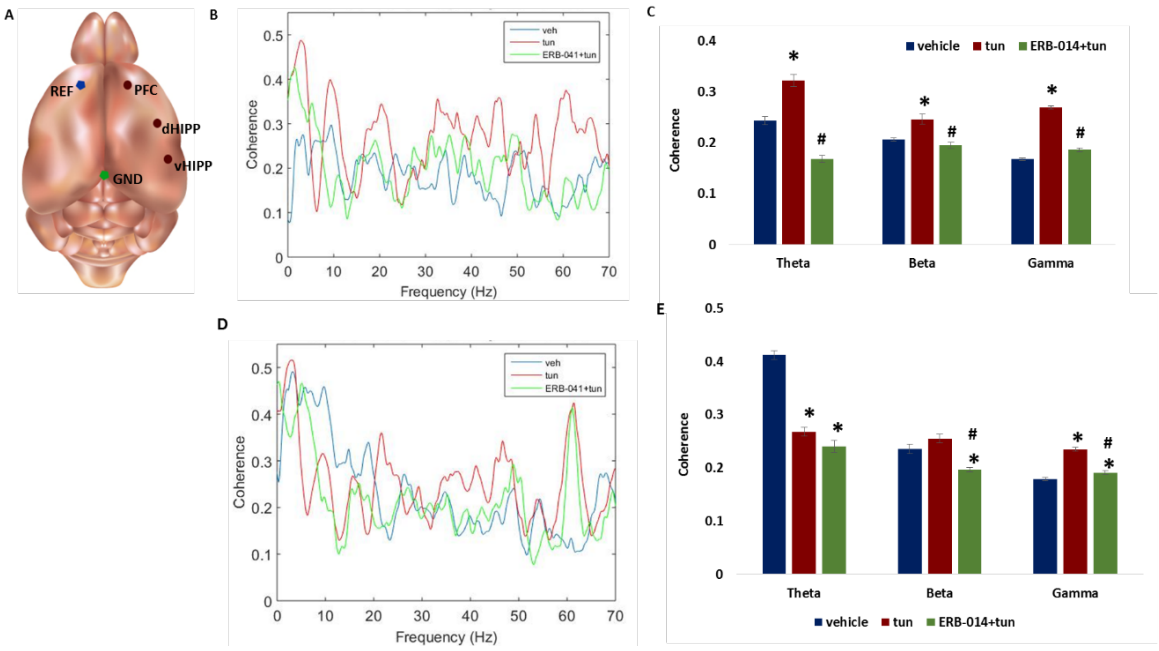

Figure 9. ERβ agonist ERB-041 attenuates ER stress-induced brain hyperconnectivity in male mice.

Adult male mice were injected intraperitoneally with tunicamycin (1mg/kg in DMSO), vehicle (DMSO), or ERB-041 (1mg/kg in DMSO, 30 minutes before tunicamycin injection) and tunicamycin (1mg/kg in DMSO). LFP recordings were performed 12 hours after injection. A) Electrode diagram, (B) Coherence v. frequency of mPFC-dorsal HIPP, (C) coherence in theta (4–12 Hz), beta (15–25 Hz) and gamma (26–70 Hz) bands in mPFC-dorsal HIPP, (D) Coherence v. frequency of mPFC-ventral HIPP, and (C) coherence in theta, beta and gamma bands in mPFC-ventral HIPP. Coherence was determined using values from 3 seconds before and 3 seconds after (for a total of 6 seconds) a novel social interaction with a stranger mouse. REF = reference electrode, mPFC = medial prefrontal cortex electrode, dHIPP = dorsal hippocampal electrode, vHIPP = ventral hippocampal electrode, GND = ground electrode. Data are expressed as mean ±s.e.m. (*n*=4-5). *p<0.05 vs. vehicle. $^{\$}$p<0.05 vs. tunicamycin. One-way ANOVA.

ERB-041 attenuates ER stress-induced activation of CHOP mRNA in mouse PFC.

It is known that CHOP is a downstream target of IRE-1/Sxbp1 pathway and is induced following ER stress (182, 197, 198). To determine whether tunicamycin-induced increase in IRE-1 pathway is accompanied by increase in CHOP levels, we examined CHOP mRNA levels in mouse PFC following tunicamycin administration. We found significant increases in CHOP mRNA levels in the PFC of mice treated with tunicamycin ($p < 0.05$; Figure 9). Moreover, ERB-041 pretreatment significantly attenuated tunicamycin-induced increase in CHOP expression ($p < 0.05$; Figure 5).

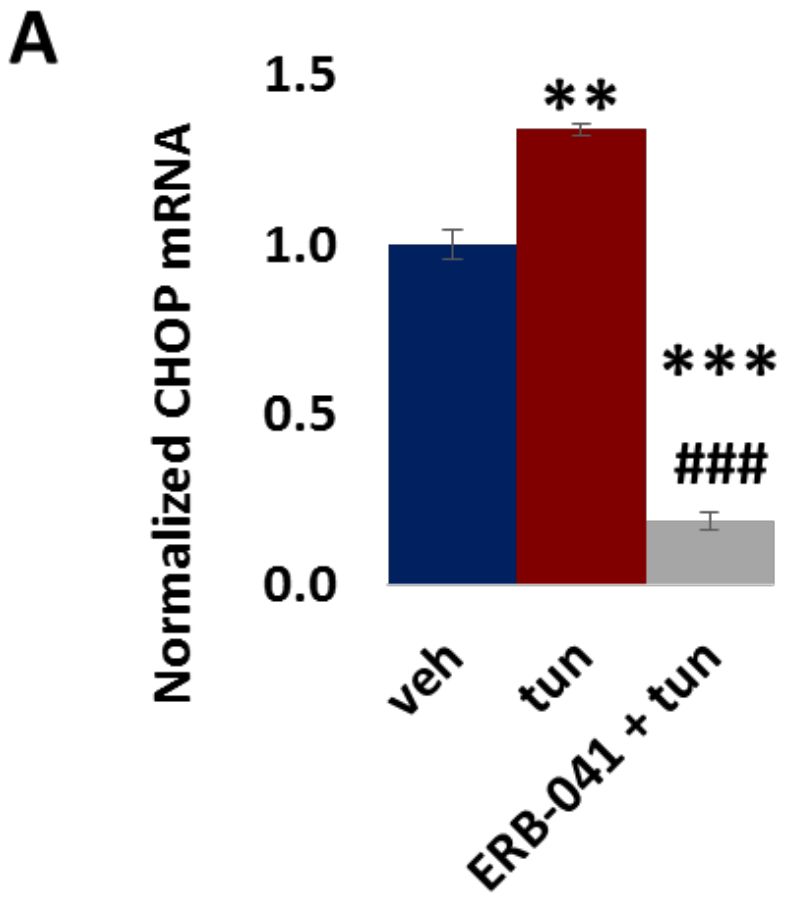

Figure 10. ERB-041 attenuates ER stress-induced increases in CHOP mRNA levels in mouse PFC.

Adult male mice were injected intraperitoneally with tunicamycin (1mg/kg in DMSO), vehicle (DMSO), or ERB-041 (1mg/kg in DMSO, 30 minutes before tunicamycin injection) and tunicamycin (1mg/kg in DMSO). mRNA levels of CHOP were determined by qRT-PCR in the mouse PFC 12 h after tunicamycin injection. The Ct values were normalized to RPS3. (*n*=4-5). Data are expressed as mean ±s.e.m. **p<0.01, ***p<0.001 vs. vehicle. $^{\$\$}$p<0.01 and $^{\$\$\$}$p<0.001 vs. tunicamycin. One-way ANOVA.

Discussion

Emerging evidence suggests that ER stress may be one potential mechanism involved in synaptic deficits and social behavioral alterations found in neuropsychiatric disorders including schizophrenia, depression and ASD (127, 187-190). In the present study, we used the glycosylation inhibitor, tunicamycin to induce ER stress (199-201) in mice to determine if induction of this cellular response results in social behavior deficits. Glycosylation is of great physiological significance since changes in glycans significantly change the structure and function of polypeptide parts of glycoproteins (202). Proper glycosylation of membrane receptors is critical for adaptive properties of the cell and affects communication between cells (203).

The lack of deficits in social behavior in female mice following tunicamycin treatment suggests that ER stress induced deficits in social behavior in mice is sex specific. The decrease in ERβ, with no change in ERα protein levels in the PFC of tunicamycin-treated mice found in this study is consistent with the increasing evidence for the role of ERβ signaling in the etiology of psychiatric disorders such as schizophrenia and ASD (9, 117, 115). The role of ERβ in neuroplasticity is further supported by a number of studies in rodents. ERβ mediates some of the effects of estrogens on anxiety, locomotor activity, fear responses, and learning behavior (19, 21, 22). ERβ knockout, but not ERα knockout mice showed defects of neuronal migration (157), and ERβ knockdown abolished E2-induced reductions in depressive behavior in mice (99, 158, 159). Moreover, administration of ERβ agonist has been shown to reduce anxiety-like behavior (159) and depressive behavior (105) in rats. In cultured cortical neurons, activation of ERβ with the

specific agonist WAY-200070 has been shown to increase spine density and PSD-95 (postsynaptic density-95) accumulation in membrane (204). Our data from experiments using ERβ agonist (ERB-041) further strengthened the role of ERβ in social behavior.

We found hyperconnectivity as indicated by increase in coherence between mPFC and dorsal hippocampus in theta, beta and gamma bands in tunicamycin-treated mice. Brain hyperconnectivity has been shown to be linked to impairments in social behavior. For example, ASD children with greater connectivity exhibited more severe impairment in the social domain (205). In addition, a number of studies have shown neural network oscillatory abnormalities such as abnormal gamma oscillations in ASD and schizophrenia (206-209). Gamma oscillations are instrumental for the synchronization of neuronal discharges in cortical networks and sensory processing (210-212). Hyperconnectivity has been observed among the frontal, temporal, and subcortical regions in gamma frequency ranges in subjects with ASD and schizophrenia (213-216). The hyperconnectivity in tunicamycin-treated mice could be a result of aberrant balance of excitation and inhibition in local neural circuits (216-218). An imbalance between excitation and inhibition has been postulated as a neurophysiological mechanism underlying the social behavior deficits (217, 218). Together, the brain-behavior relationship found in mice following ER stress suggests that aberrant functional connectivity may underlie the deficits in social behavior.

How does ERB041 protect the mice from ER stress-induced ASD-like behavior in mice? Activation of ER stress transducer IRE1α is known to produce the spliced form of XBP1

(219). We investigated whether ERB041 treatment is associated with inhibition of ER stress-induced activation of IRE1. Indeed, pretreatment with ERB-041 significantly decreased the ER stress-induced increase in IRE1 activation in mouse PFC suggesting that inhibition of IRE1/XBP1 signaling could a potential mechanism involved in ERβ signaling mediated rescue of deficits in social behavior (Figure 10). Moreover, we found significant reduction in ER stress-induced increase in CHOP mRNA levels in mice pretreated with ERB-041. ER stress has been known to activate CHOP, which induces the expression of proinflammatory cytokines such as IL-23 (197). In addition, ER stress activates NLRP3 inflammasomes via thioredoxin-interacting protein (TXNIP), leading to increases in proinflammatory cytokine levels (219, 220). We found significant attenuation in tunicamycin-induced increase in CHOP mRNA levels in mice pretreated with ERB-041. ERB-041 has been shown to inhibit inflammatory signaling in many rodent models of inflammatory diseases such as bowel disease, adjuvant-induced arthritis, and experimentally-induced sepsis (221). Moreover, estrogens have been shown to modulate all subsets of T cells that include CD4+ (Th1, Th2, Th17, and Tregs) and CD8+cells (222). In addition, estrogens promote the expansion and frequency of Treg cells, which play a critical role in downregulating immune responses (223, 224). It is important to note that ERβ is present in neurons and astrocytes (225). Although our study found a protective role of ERB-041 in ER stress-induced inflammation and social behavior, the cell type/s involved in ERβ signaling needs further investigation.

Collectively, these studies suggest that ERβ signaling plays an important role in ER-stress induced changes in brain connectivity and social behavior. Although the above data

on the effects of pharmacological induction of ER stress on social behavior deficits in adult mice are interesting, further studies are warranted to understand the role of ER stress during neurodevelopment on social behavior. Also, it is important to find out whether ER stress induces changes in connectivity between PFC and brain regions other than hippocampus such as amygdala which is also implicated in social behavior. At the translational level, as selective ERβ agonists are relatively free of estrogenic side effects and yet have demonstrated estrogen-like effects in the brain including improvement in cognitive performance and social behavior, the selective ERβ modulators would provide a new strategy to improve social behavior deficits seen in many neuropsychiatric disorders.

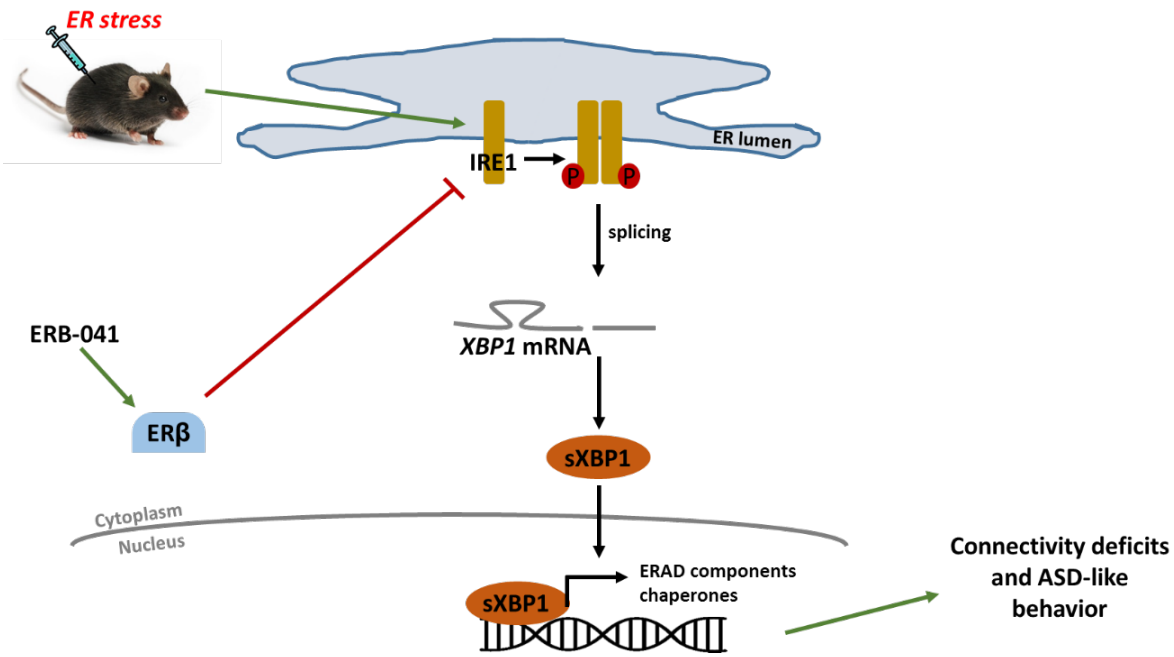

Figure 11. Schematic Diagram of ERβ regulation of the IRE/XBP1 pathway.

ER stress leads to the activation of IRE1 that promotes splicing of XBP1 mRNA. This leads to increased levels of the transcription factor sXBP1, which triggers the expression of UPR genes that induces alterations in brain connectivity and ASD-like behavior. The activation of ERβ signaling inhibits IRE1 signaling, downregulates sXBP1 and attenuates ER stress-induced ASD-like phenotype.

1. Altered expression of endoplasmic reticulum stress---related genes in the middle frontal cortex of subjects with autism spectrum disorder.

Abstract

The endoplasmic reticulum (ER) is an important organelle responsible for the folding and sorting of proteins. Disturbances ER homeostasis can trigger a cellular response known as the unfolded protein response (UPR), leading to accumulation of unfolded or misfolded proteins in the ER lumen called ER stress. A number of recent studies suggest that mutations in ASD susceptible synaptic genes induce ER stress. However, it is not known whether ER stress-related genes are altered in the brain of ASD subjects. In the present study, we investigated the mRNA expression of ER stress-related genes (ATF4, ATF6, PERK, XBP1, sXBP1, CHOP and IRE1) in postmortem middle frontal gyrus of ASD and control subjects. RT-PCR analysis showed significant increases in the mRNA levels of ATF4, ATF6, PERK, XBP1, sXBP1, CHOP and IRE1 in the middle frontal gyrus of ASD subjects. In addition, we found a significant positive association of sXBP1 mRNA with the diagnostic score for abnormality of development in ASD subjects. These results, for the first time, provide the evidence of the dysregulation of ER stress genes in the brain of subjects with ASD.

Introduction

Autism spectrum disorder (ASD) is among the most devastating neurological disorders of childhood with a prevalence of about 1 in 68 children (226). It is characterized by social interaction deficits, difficulties in both verbal and nonverbal communication, repetitive

behaviors, and obsessive tendencies. In addition, neuroimaging studies have provided evidence for aberrant functional and structural connectivity between cortical regions in individuals with ASD (175, 227). While there is a clear genetic predisposition to ASD, known genetic variants can account for no more than 5-15% of all cases and there is emerging evidence that epigenetic factors also contribute to the molecular pathology of the illness (4, 228).

The endoplasmic reticulum (ER) is an important organelle responsible for the folding and sorting of proteins. Disturbances in redox or calcium homeostasis in the ER caused by pathophysiological or environmental stimuli can trigger a cellular response known as the unfolded protein response (UPR), leading to accumulation of unfolded or misfolded proteins in the ER lumen called ER stress (129). Three ER-resident proteins have been identified as sensors of ER stress: IRE1 (inositol-requiring protein 1), PERK [PKR (double-stranded-RNA-dependent protein kinase)-like ER kinase] and ATF6 (activating transcription factor (128; 113). IRE1 is a type 1 transmembrane serine/threonine receptor protein kinase which functions as a sensor for misfolded/unfolded proteins in the ER lumen. Activated IRE1 induces the splicing of *XBP1* (X-box-binding protein 1) mRNA by cleaving off its intron (181; 182). PERK is a type 1 transmembrane protein kinase that transmits stress signals in response to the perturbation of protein folding (183). When activated, PERK phosphorylates the α subunit of eIF2 (eukaryotic initiation factor 2) leading to the translation of ATF4 and activation of the CHOP promoter (183, 184). ER stress activates ATF6 by translocating it from the ER to Golgi complex, where it is cleaved by the Golgi-resident serine proteases S1P and S2P (site 1 and site 2 proteases

respectively) (185, 186) resulting in the activation of the transcription of UPR targets such as GRP78, CHOP and XBP1 (182; 186). The UPR is generally a pro-survival mechanism, mediated by translation arrest and the induction of a number of transcription factors and chaperone proteins that function to restore ER homeostasis and help the cells adapt to ER stress conditions. However, when ER stress is prolonged or the degree of ER stress is too severe, UPR signaling can initiate programmed cell death by activating stress-induced pro-apoptotic factors (130, 131).

A number of recent studies have suggested an important role of endoplasmic reticulum (ER) stress in the pathophysiology of ASD (7, 187, 127, 128). In particular, genetic variations in several synaptic genes implicated in ASD have been shown to induce ER stress genes. Over-expression of Neuroligin3 mutant has been shown to activate the UPR downstream of the stress sensors ATF6, IRE1, and PERK (229). Mutated GPR85 resulted in ER stress and impaired dendrite formation of hippocampal neurons (230). In addition, mutated cell adhesion molecule-1 (CADM1) as well as neuroligin (NLGN) 3 (R451C) have been shown to upregulate CHOP expression (187). Although these studies are interesting, it is still not known whether ER stress-genes are altered in the brain of ASD subjects. In the present study, we examined the gene expression of ATF4, ATF6, PERK, XBP1, Sxbp1, CHOP and IRE1 in postmortem middle frontal gyrus of ASD and control subjects. A number of studies from neurocognitive as well as neuroimaging studies as well as reports from our laboratory have shown an important role of middle frontal gyrus in the pathophysiology of ASD (117, 149-151). We hypothesized that the expression of ER stress-related genes is impaired in ASD.

Methods

Ethics statement

The Augusta University Institutional Review Board has deemed this study exempt from full review due to the use of de-identified human postmortem brain samples, with no possibility to track back the identity of the donors. Human postmortem samples are from the NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland with ethical permission granted by the institutional review boards of the University of Maryland. There was no involvement of animal studies in this paper.

Postmortem samples.

Postmortem tissues from middle frontal gyrus of control (n=12; M/F = 11/1) and ASD (n=13; M/F = 13/0) subjects were obtained from NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, MD, USA. Control and ASD subjects were age- and sex-matched. **Table 3** shows a detailed description on the demographics of samples**.** The individual scores for each of the symptomatic domains, Autism Diagnostic Interview-Revised (ADI-R) were obtained from the brain bank website. The information on ADI-R was available for 9 out of 13 subjects with ASD. We did not find any significant difference in confounding variables such as PMI, refrigeration interval, age at death, RNA integrity, and brain pH between ASD and control subjects.

Quantitative reverse transcriptase PCR (qRT-PCR).

RNA was purified using a commercially available kit (SV RNA Isolation, Promega, Madison, WI, USA), qRT-PCR was performed on a MasterCycler (Eppendorf, Hamburg, Germany) using a SuperScript III Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen, Carlsbad, CA, USA). Gene-specific primers were synthesized by Integrated DNA Technologies. Primers used are given in **Table S1**. Ct values of genes of interest were normalized to that of housekeeping gene, 18S.

Statistics.

We used Multivariate Analysis of Covariance (MANCOVA) to examine differences in the mRNA levels of ER stress genes in the postmortem samples of people with ASD relative to control samples including—ATF-4, ATF-6, PEK, XBPI, sXBPI, CHOP, and IRE1. We examined group differences in mRNA levels while considering age, storage time, post mortem interval (PMI), pH, and RNA integrity for inclusion as possible covariates in the MANCOVA. Following an initial evaluation of the covariates, age, storage time, and sample pH were retained as covariates given that they possessed at least small associations—correlations of 0.20 and higher—with one or more of the protein signals. Partial Eta-square (η^2_p) was computed as a measure of effect size difference and p-values less than 0.05 were flagged as statistically significant. All analyses were performed using SPSS Statistics 20 software (IBM).

Results and Discussion

The postmortem samples included 13 individuals with ASD and 12 unaffected controls. Age, storage time, and sample pH were the only covariates that produced at least small associations with one or more of the mRNA levels of ER stress genes and storage time often produced significant associations (**Table 4**). In the overall multivariate model, sample pH remained the only statistically significant predictor of the ER stress genes [Wilk's $\lambda = 0.399$, $F(7, 14) = 3.01$, $p < 0.05$, $\eta^2_p = 0.60$]. Examination of the univariate between subject effects showed that in particular, sample pH had significant effects on XBPI mRNA. Age [Wilk's $\lambda = 0.495$, $F(7, 14) = 2.04$, $p > 0.05$, $\eta^2_p = 0.51$] and storage time [Wilk's $\lambda = 0.620$, $F(7, 14) = 1.23$, $p > 0.05$, $\eta^2_p = 0.38$] were not significant predictors in the model.

An examination of the full multivariate model with age, storage time, and sample pH as covariates showed that ASD status was a significant predictor of mRNA levels of ER stress genes [Wilk's $\lambda = 0.015$, $F(7, 14) = 130.12$, $p < 0.001$, $\eta^2_p = 0.985$, Observed Power = 1.00]. An examination of the univariate between-subject effects showed that ASD status was associated with an increased mRNA levels of ER stress genes (**Figure 11**), suggesting that the overexpression of these genes may be implicated in ASD. **Table 5** summarizes the association of ER stress genes with domains of the ADI-R. We found a significant positive association of sXBP1 mRNA with the diagnostic score for abnormality of development.

Table 3. Comparison of Autism Spectrum Disorder (ASD) and Control Samples on Evaluated Covariates

Covariate	ASD	Control	*F* (1, 23)	*p*
M (SD)				
Age	11.80 (5.80)	10.83 (5.01)	0.20	p>0.05
PMI	19.00 (10.01)	14.50 (8.17)	1.50	p>0.05
Storage Time	2828.77 (1434.06)	4400.42 (2366.92)	4.11	p>0.05
Sample pH	6.11 (0.26)	5.95 (0.22)	2.73	p>0.05
RNA Integrity	6.84 (1.92)	5.81 (2.48)	1.36	p>0.05

Table 4. Correlation between covariates and ER stress genes

	ATF4	ATF6	PERK	XBP1	sXBP1	CHOP	IRE1
Storage Time	-.360	-.407*	-.404*	-.422*	-.355	-.340	-.352
Sample pH	.174	.215	.157	.113	.168	.223	.169
RNA Integrity	.204	.196	.122	.068	.184	.201	.181
Age	.310	.262	.306	.192	.326	.301	.255
PMI	.114	.165	.122	.109	.152	.159	.159

ATF4 =Activating Transcription Factor 4; ATF6 = Activating Transcription Factor 6; PERK = Protein Kinase-like Endoplasmic Reticulum Kinase; XBPI = X-box Protein I; sXBPI = Spliced X-Box-Protein 1; CHOP = CCAAT-enhancer-binding protein homologous protein; IRE1 = Inositol Requiring Enzyme 1; PMI = Postmortem Interval; *$p<0.05$

The current knowledge regarding the role of ER stress in the pathophysiology of ASD is mainly based on data from autism-associated mutations in synaptic genes such as neuroligin3, *CNTNAP2* and *CADM1* (127, 187, 229, 231). Our data provide the first evidence of altered ER stress genes in the brain of ASD subjects. Cotranslational modifications such as N-linked glycosylation and formation of disulfide bonds, facilitate proper folding of nascent polypeptides in the ER. Glycosylation is of great physiological significance since changes in glycans significantly change the structure and function of polypeptide parts of glycoproteins (202). Proper glycosylation of membrane receptors is critical for adaptive properties of the cell and affects communication between cells (203). Altered glycosylation could contribute to the pathophysiology of ASD, and indeed a number of mutations in enzymes involved in glycosylation are found in people with autism (232).

We found significant increases in ATF-4, ATF-6, PEK, XBPI, sXBPI, CHOP, and IRE1 Mrna levels in the middle frontal gyrus of ASD subjects. Among these molecules, CHOP is known to interact with the heterodimeric receptors $GABA_{B1a}R/GABA_{B2}R$ and inhibits the formation of heterodimeric complexes resulting in the intracellular accumulation and reduced cell surface expression of receptors (233). Interestingly, decreased levels of $GABA_{B1}R$ and $GABA_{B2}R$ have been found in the brain of ASD subjects (234). What are the downstream mechanism mediating ER stress-induced changes in CNS function? One potential mechanism is inflammation. Accumulating evidence suggest that pathways activated by the ER stress response induce inflammation. When activated, all three sensors of the unfolded protein response (UPR), PERK, IRE1, and ATF6, participate in

upregulating inflammatory processes. It is known that PERK and IRE1 activation can interfere with NFkB inhibitory signals, thereby promoting a proinflammatory response (31, 32). In addition, CHOP has been shown to induce the expression of proinflammatory cytokines such as IL-23 (197). Moreover, ER stress activates NLRP3 inflammasomes via thioredoxin-interacting protein (TXNIP), leading to increases in proinflammatory cytokine levels (219, 220). In this regard, our earlier studies using the same tissue samples of the present study found increased levels of proinflammatory cytokines, IL-1β and IFN-γ in the middle frontal gyrus of ASD subjects (235, 236).

We found that sXbp-1 mRNA levels are positively associated with the diagnostic score for abnormality of development. It has been shown that overexpression of sXbp-1 in neurons improves performance in the memory flexibility and contextual fear-conditioning tests in mice (237). Moreover, overexpression of XBP1s resulted in improved LTP, synaptic transmission and increased BDNF expression in mouse hippocampus (237). Interestingly, a number of studies have shown increased levels of BDNF in subjects with ASD (238). Although BDNF plays a critical role in neurodevelopment (239), it is important to determine whether excessive BDNF levels can lead to developmental abnormalities as seen in ASD.

The present data was collected in a relatively smaller number of study subjects, which needs further investigation using large samples before a conclusion can be drawn. To further establish a definitive role of ER stress in ASD pathophysiology, the following questions still need to be addressed: (1) Is ER stress in ASD of neurodevelopmental

origin? (2) Are there factors other than mutant synaptic proteins that can trigger ER stress leading to ASD phenotype? (3) Is inflammation triggering ER stress or is ER stress triggering inflammation leading to ASD phenotype? (4) Does ER stress induce changes in neural connectivity between key brain regions implicated in ASD pathophysiology? Future studies addressing the above questions might lead to a better understanding of the pathophysiology and provide new avenues of treatment of this disorder.

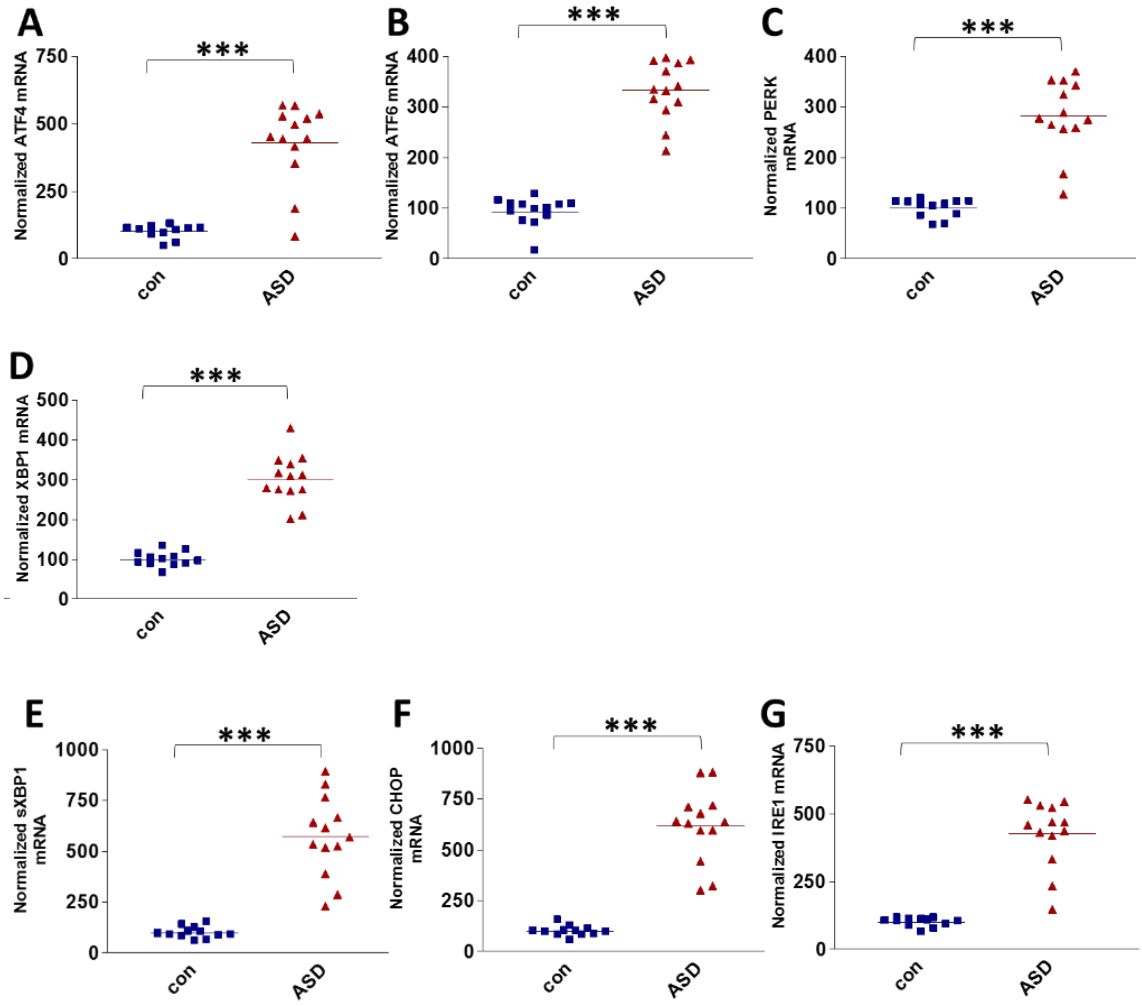

Figure 12. Increase in mRNA levels of ER stress genes in the middle frontal gyrus of ASD subjects.

mRNA levels of ER stress genes were determined by qRT-PCR in the middle frontal gyrus of ASD (*n*=13) and control (*n*=12) subjects. The Ct values were normalized to the 18S. A) ATF4, B) ATF6, C) PERK, D) XBP1, E) sXBP1, F) CHOP, and G) IRE1. ***p<0.0001 vs. controls.

Table 5. Correlations of ER stress mRNAs with ADI-R scores

	ATF4	ATF6	PERK	XBP1	sXBP1	CHOP	IRE1
ADI---R Social Interaction	0.301	0.273	0.207	0.126	0.272	0.38	0.315
ADI---R Verbal Communication	---0.161	---0.223	---0.046	0.153	---0.138	0.013	---0.109
ADI---R Non--- Verbal Communication	0.401	0.37	0.489	0.434	0.343	0.365	0.328
ADI---R Stereotyped Behavior	---0.479	---0.374	---0.542	---0.608	---0.367	---0.509	---0.439
ADI---R Abnormality of Development	0.595	0.607	0.527	0.256	0.694*	0.626	0.561

*p<.05

Acknowledgements

Human postmortem samples were obtained from the NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, MD, USA. The Bank is funded by NIH Contract No. #HHSN275200900011C, Ref. No. NO1-HD-9-0011. The authors would like to acknowledge the research support from US National Institute of Mental Health grant R01 MH 097060 (A.P.).

Conflicts of Interest

There are no conflicts of interest to report.

IV. UNPUBLISHED RESEARCH

1. ERβ protects against Transglutaminase 2-induced ASD-like behavior in mice.

Abstract

Background: Although a number of recent studies have suggested the role of endoplasmic reticulum (ER) stress in the pathophysiology of autism spectrum disorder (ASD) in humans, the underlying mechanism(s) is not known. Transglutaminase 2 is a calcium dependent enzyme that is known to be activated following ER stress. TG2 is located on the chromosome 20q11.2, a region associated with susceptibility to ASD. Moreover, a gene expression study has reported higher TG2 expression in the frontal cortex of ASD subjects. In the present study, we investigated whether TG2 mediates ER stress-induced ASD-like behavior in mice.

Methods: Adult male mice were injected intraperitoneally with ER stress inducer, tunicamycin (1mg/kg) and subsequently examined in social interaction, marble burying, grooming, ultrasonic vocalization and open field tests. The role of transglutaminase 2 was examined using TG2 overexpressed mice, lentiviral and well as pharmacological approaches. Human postmortem samples included middle frontal gyrus samples from ASD subjects (n = 13) and age and sex-matched controls (n = 12).

Results: Tunicamycin treatment induced deficits in social behavior, increase in repetitive behavior and communication deficits. We found significant increase in TG2 mRNA and protein levels in mouse PFC following ER stress. Mice overexpressing TG2 in neurons showed ASD-like behavior. Moreover, TG2 overexpression in mouse PFC using lentiviral particles induced ASD-like behavior. Inhibition of TG2 using cysteamine or TG2 lentiviral particles in mouse PFC attenuated ER stress-induced ASD-like behavior in mice. Reduced estrogen receptor beta (ERβ), but not estrogen receptor alpha (ERα) protein levels were found in PFC of male mice overexpressing TG2. Moreover, ER beta agonist significantly attenuated ER stress-induced ASD-like behavior in mice. Increased mRNA and protein levels of TG2 were also found in the middle frontal gyrus of ASD subjects as compared to controls.

Introduction

ASD is one of the most prevalent neurodevelopmental disorders. As of 2016, 1 in 68 children in America were diagnosed with ASD (226). It is characterized by a set of core symptoms including social interaction deficits, difficulties in verbal and nonverbal communication, repetitive or injurious behavior, and obsessive tendencies. ASD lacks specific pathological hallmarks and thus remains one of the least understood neurodevelopmental disorders. Genetic variants account for no more than 5-15% of all ASD cases, suggesting that epigenetic factors, which would be easier to target with novel therapeutics, contribute to the molecular pathology of ASD (4, 228).

A number of recent studies suggest an important role of Endoplasmic Reticulum (ER) stress in the pathophysiology of ASD (7, 127, 128, 187**).** In ASD, ER stress arising from genetic variations in several synaptic genes implicated in ASD (such as neuroligin, neurexin, *CNTNAP* [contactin-associated protein] and *CADM1* [cell-adhesion molecule 1]) have been shown to cause impaired synaptic function and signal transduction (127, 128). Moreover, relatively low levels of ER stress have been implicated in altered membrane trafficking of the synaptic functional molecules such as GABA receptors leading to ASD pathophysiology (127). ER stress can alter the delicate balance of post-translational modifications occurring in the cell, which can lead to the improper production or degradation of functional proteins.

Studies have shown that ER stress induces the activity of TG2, a Ca2+ dependent enzyme involved in neural development and functioning (15, 16). TG2 has many functions, some of which are transamidation, deamidation, histone modifications, and posttranslational modifications of proteins (23, 24) TG2 is located on the chromosome 20q11.2, a region associated with susceptibility to ASD (17). Moreover, a gene expression study has reported higher TG2 expression in the frontal cortex of ASD subjects (15). A significant increase in serum levels of IgA antibodies to TG2 has also been reported in children with ASD (18).

Although ER stress is implicated in ASD, the underlying mechanism(s) is not known. Our previous study (240) showed that inducing ER stress in mice by tunicamycin administration induces ASD-like behavioral deficits in male mice. We utilized this model

to study the role of TG2 in ER stress-induced ASD-like behavior in mice. We manipulated TG2 expression via lentiviral transduction and pharmacological inhibition. Tunicamycin treatment induced mRNA and protein levels of transglutaminase 2. Inhibition of TG2 via viral particle or pharmacological treatment (cysteamine) attenuated behavioral deficits induced by tunicamycin. Reduced estrogen receptor beta (ERβ), but not estrogen receptor alpha (ERα) protein levels were found in PFC of male mice overexpressing TG2. This is consistent with data showing significant reduction in ERβ, but not ERα in the medial frontal gyrus of ASD subjects as compared to age- and sex-matched controls (Crider et al., 2014). ASD subjects showed increased mRNA and protein levels of TG2 in the same brain region where reduced ERβ mRNA and protein levels were found. TG2+/+ mice were then treated with an estrogen receptor beta agonist, ERB-041, to determine if ERβ could reverse ASD-like phenotype in TG2+/+ mice. This rescued ASD-like behavior in TG2 +/+ mice, suggesting that ERβ is involved in TG2-induced ASD-like behavior in male mice.

Materials and Methods

Animals.

Adult (8-10 week old) C57BL/6J male and female wild-type or Transglutaminase 2 overexpressing (TG2+/+) littermates were bred in-house at Augusta University Laboratory Animal Services facility. The original breeding pair was from Dr. Janusz Tucholski (University of Alabama at Birmingham). Wild-type mice used for lentiviral injections and pharmacological studies were purchased from from Jackson Laboratory (Bar Harbor, Maine, USA). Mice were housed in groups of 4-5 mice in standard

polypropylene cages in 12-h light-dark cycle in compliance with the US National Institute of Health guidelines and approved by Augusta University animal welfare guidelines.

Drug treatment.

Mice were injected intraperitoneally with 1mg/kg tunicamycin (catalog #T7765; Sigma, St. Louis, Missouri) dissolved in DMSO. This dose was selected based on previous studies showing that it actively induced ER stress in mice (24). The selective ERβ-specific agonist, (ERB-041; catalog #PZ0183; Sigma, St. Louis, Missouri; 1 mg/kg) or vehicle (DMSO) was administered 30 minutes before tunicamycin injection. The TG2 antagonist (cysteamine; Sigma, St. Louis, Missouri; 150 mg/kg) or vehicle (distilled water), was administered 30 minutes before tunicamycin injection. Mice were monitored for adverse effects of treatments. Behavioral experiments were performed 12 hours after tunicamycin injection.

Stereotaxic Injection of Lentivirus.

TG2 siRNA (m) Lentiviral (LV) Particles and its control siRNA LV particles were purchased (abm Incorporated, Richmond, BC). TG2 siRNA lentiviral particles are a proprietary pLenti-Combo packaging mix with up to 10^{10} IU/ml. 2.0 ul of lentiviral particles per injection were infused bilaterally into mouse PFC (coordinates: x ±.5 mm mediolateral, y +1.0 mm posterioanterior, with respect to bregma at 0, and z +1.0 mm dorsoventral from dura at 0). For this procedure, mice were held on a stereotaxic frame

(Kopf Instruments Inc) and an automated microinjector (Stoelting Co) was used to inject the particles at a rate of 0.2 µl/min at each site (193).

Behavior experiments.

Behavioral testing was performed in a room with consistent background noise and ambient lighting approximately 25-30 Lux (lumen/m2) unless otherwise noted. Behavioral room temperature and pressure is monitored and kept constant. Animals were transferred to behavioral rooms in their home cages at least 1 hour prior to testing for habituation to the testing room. All behavioral experiments were scored blind to treatment.

Three Chamber Test.

This test was performed to measure sociability and social deficits. The test mouse was placed in a box with 3 chambers. Each chamber was 19 cm x 45 cm x 22 cm and the dividing walls were made from clear Plexiglas®, with openings on each wall for free access to the other two chambers. Two identical wire containers that were large enough to house a single mouse were placed vertically inside the apparatus with one in each side chamber and weighted down. The test mouse was habituated to the apparatus for 5 minutes while freely exploring. After the habituation period, the stranger mouse was placed in one of the wire containers while the test mouse was still allowed to freely move outside of the container. The wire containers allowed air exchange between the interior and exterior, but the holes were small enough to prevent direct physical contact between

the stranger mouse and test mouse. The free test mouse was allowed to interact through the wire container with the stranger mouse for 5 minutes. During this time, time spent in chambers (mouse, center, and empty) was recorded by an examiner with a stopwatch. The mouse chamber was defined as the chamber containing the wire container with the stranger mouse inside. The empty chamber was the chamber containing an empty wire container and the center chamber was the buffer chamber between the two. The stranger mouse was a mouse of similar age, same sex, and similar weight as the test mouse.

Reciprocal Social Interaction Test.

This test was performed to measure sociability and social deficits. The test mouse was placed in a box measuring 58 cm x 45 cm x 22 cm and allowed to habituate for 5 minutes. A stranger mouse was then placed in the chamber and the test mouse was allowed to freely interact with the stranger mouse. Time spent interacting (initiated by the test mouse) was recorded by an examiner with a stopwatch and counter. Interaction time is defined as time spent sniffing, in close contact, grooming, or licking of the stranger mouse by the test mouse. The examiner was blinded to the treatment groups and sex. The stranger mouse was a mouse of similar age, same sex, and similar weight as the test mouse.

Grooming Behavior.

Each mouse was placed singly in a standard polypropylene cage without bedding and with a lid. The freely-moving mouse was habituated to the cage for 5 minutes. After the habituation period, the mouse was allowed to freely move around the cage for 10

minutes. The amount of time and number of times (bouts) that the mouse spent grooming the face, tail, limbs, and body was recorded by an examiner with a stopwatch and counter. The examiner was blinded to the treatment groups and sex. Grooming behavior serves as a measure of repetitive behavior (238, 239).

Marble bury test.

Each mouse was placed singly in a lidded standard polypropylene cage with approximately 2 inches of corncob bedding and 10 equidistantly placed marbles. All marbles were of the same size and texture. The test mouse was left undisturbed in the cage for 30 minutes and allowed to freely move about the cage and dig in the bedding. The number of buried marbles out of the total of 10 was counted and recorded by the examiner. The examiner was blinded to the treatment groups and sex. Marble bury test was used as a measure of repetitive behavior (196, 240, 243).

Open field test.

The open field test was performed as an indicator of overall mobility and health of the animal. Mice were placed in an open field chamber and total distance traveled (cm) was measured over a 15 minute period**.** The open field chamber measured 40 × 40 × 40 cm and was constructed of white opaque Plexiglas®. A video camera was fixed over the chamber by an adjacent rod for video tracking. Ethovision XT 10 (Noldus Information Technologies Inc, USA) software was used for analysis. Trial totals for all parameters were taken.

Ultrasonic Vocalizations.

Ultrasonic vocalizations were recorded and analyzed for purposes of assessing same sex social communication. Mice were habituated to the testing chamber for 5 minutes, then introduced to an intruder mouse (stranger mouse of the similar age, same sex, and similar weight as the test mouse) for 5 minutes. The intruder mouse was then removed from the testing chamber and the calls of the test mouse were recorded for 5 minutes. The calls were recorded using Avisoft Recorder USGH (Avisoft Bioacoustics, Glienicke, Germany, RRID:SCR_014436). The files were analyzed using SASLab Pro (Avisoft Bioacoustics, Glienicke, Germany, RRID:SCR_014438) and an FFT (fast Fourier transform) was performed using the following settings: sampling rate: 250 Hz, FFT-length of 512 points, time window overlap of 75% (100% frame hamming window). Frequency resolution was 488 Hz, time resolution was 1ms, and the lower cut off frequency was 20 kHz. Mean duration of calls, total duration of calls, mean peak amplitude, and vocalizations per minute were analyzed for each mouse and group averages were plotted.

Western blotting.

Animals were anesthetized with isoflurane and sacrificed by cervical dislocation. PFC tissue was homogenized using ceramic beads in a tissue lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1.0% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 2 mM EDTA, 6 µM PMSF, and 1.0% Triton X-100 supplemented with protease inhibitor cocktail (Sigma, St. Louis, Missouri). The homogenate was centrifuged

at 13,000 rpm for 10 min at 4°C. The supernatant was placed in a clean tube and used for protein estimation by the bicinchoninic acid method (BCA Protein Assay Kit, Sigma, St. Louis, Missouri). Samples (30 µg total protein) were subjected to SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was blocked for 1 hour in PBS with Tween 20 and 5% non-fat dry milk followed by overnight incubation with a primary antibody. . Blots were incubated in the appropriate primary antibody specific for

TG2 (Cell Signaling), ERβ (Abcam 3576; 1:1,000), ERα (Santa Cruz 71064; 1:1,000), β-Actin (Sigma A4700; 1:20,000), GAPDH (Cell Signaling 5174; 1:14,000), or Tubulin (Cell Signaling 2146; 1:12,000) and developed with the SuperSignal West Pico Chemiluminescent substrate system (Thermo Fisher Scientific). Optical densities of the bands were analyzed using ImageJ (NIH). Protein levels were normalized to tubulin, GAPDH, or β-actin levels then expressed as fold change from control animals. For figure panels, contrasts have been adjusted linearly for easier viewing of bands.

Quantitative reverse transcriptase PCR (qRT-PCR).

RNA was purified using a commercially available kit (SV RNA Isolation, Promega, Madison, WI, USA), qRT-PCR was performed on a MasterCycler (Eppendorf) using a SuperScript III Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen, Carlsbad, CA, USA). Gene-specific primers were synthesized by Integrated DNA Technologies. Primers used are given in Table 7. Ct values of genes of interest were normalized to that of two housekeeping genes (actin and GAPDH).

Postmortem samples.

Postmortem tissues from frontal cortex of control (n=12; M/F = 11/1) and ASD (n=13; M/F = 13/0) subjects were obtained from NICHD Brain and Tissue Bank for Developmental Disorders (University of Maryland, Baltimore, MD, USA). Control and ASD subjects were age- and sex-matched. Table 6 shows a detailed description on the demographics of samples**.** The individual scores for each of the symptomatic domains, Autism Diagnostic Interview-Revised (ADI-R) were obtained from the brain bank website. The information on ADI-R was available for 9 out of 13 subjects with ASD. We did not find any significant difference in confounding variables such as PMI, refrigeration interval, age at death, RNA integrity, and brain pH between ASD and control subjects.

Statistics.

Sample sizes in mouse studies were not predetermined using statistical methods, but are similar to those reported in previous publications (193-195). For mouse studies, data were analyzed using two-tailed Student's t-tests (for two-group comparisons) or one-way or two-way Analysis of Variance (ANOVA; for multiple-group comparisons). $p < 0.05$ was considered significant. Post hoc analyses were carried out using Bonferroni's test. All analyses were performed using GraphPad Prism or SPSS Statistics 20 software (IBM).

Table 6. Demographic details of subjects

Variable	Control	ASD/Autism
Age	11.70 ± 1.584	11.80 ± 1.609
PMI	14.46 ± 2.171	19.00 ± 2.776
Gender (F/M)	1/12	0/13
Storage (days)	4287 ± 638.7	2829 ± 397.7
RIN	5.55 ± 0.709	6.84 ± 0.531
pH	5.95 ± 0.058	6.11 ± 0.072

Table 7. List of human primers

Gene	Primer sequence (5’ to 3’)
TG2-RP	GAAACCCCTAAATAACGACTACGATA
TG2-FP	GGGTTATAAGTTAGCGTCGTTTTTC
GAPDH-FP	GAG TCA ACG GAT TTG GTC GT
GAPDH-RP	TTG ATT TTG GAG GGA TCT CG
Actin-FP	GGA CTT CGA GCA AGA GAT GG
Actin-RP	AGC ACT GTG TTG GCG TAC AG

Results

ER stress induces TG2 expression in frontal cortex, but not in hippocampus.

TG2 is known to be induced by ER stress, so we wanted to determine if TG2 was increased in the brain of mice treated intraperitoneally with tunicamycin, a drug that induces ER stress. Tunicamycin treated mice showed significantly higher levels of TG2 in prefrontal cortex (Figure 12a) than vehicle treated mice. TG2 was not changed in the hippocampus of tunicamycin treated mice as compared to vehicle treated mice (Figure 12b).

TG2 mediates ER stress-induced ASD-like behavior in mice.

In order to determine if TG2 is necessary for the ER stress-induced behavioral changes seen in previous studies (240), mice were treated with cysteamine, a known inhibitor of TG2. Behavioral tests were performed according to the schematic diagram (Figure 13a). Mice that were injected with tunicamycin interacted significantly less with the stranger mouse than mice injected with vehicle or those pretreated with cysteamine ($p < 0.05$; Figure 13b). To test whether ER stress induces obsessive and repetitive behaviors, marble burying and grooming tests were performed in mice. Though originally used as a measure of anxiety, marble bury test has been shown to measure repetitive behaviors (196, 240, 243).

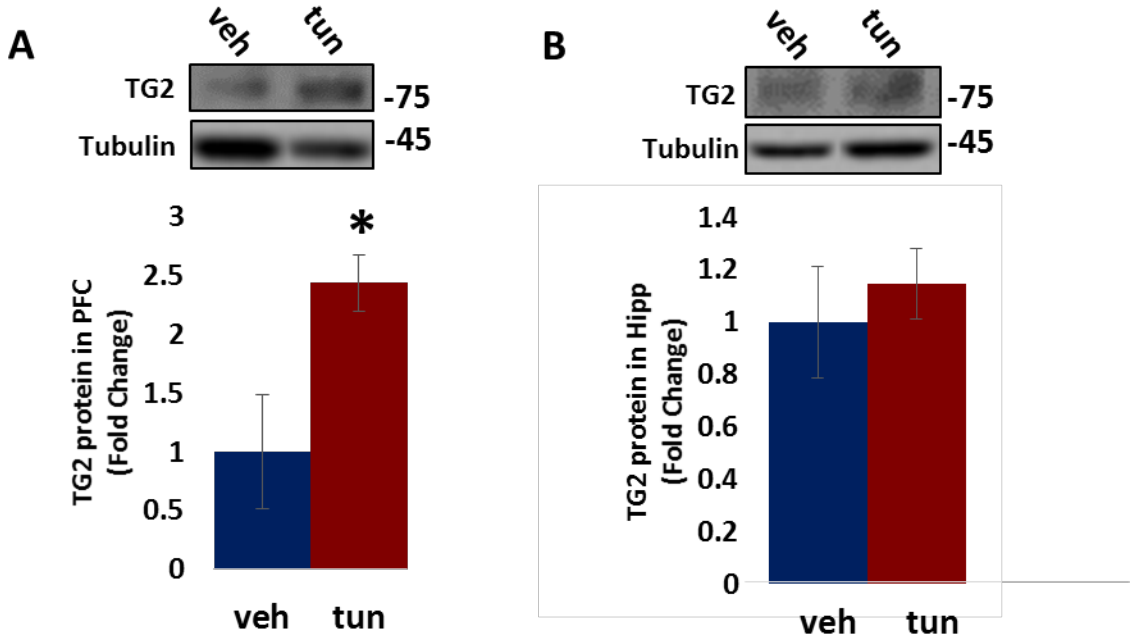

Figure 13. ER stress induces TG2 expression in frontal cortex, but not in hippocampus.

A) Representative blot and densitometric quantification of TG2 in PFC normalized to tubulin. B) Representative blot and densitometric quantification of TG2 in hippocampus normalized to tubulin. *p<0.05.

This test has been used as a measure for obsessive/repetitive behavior in many established mouse models of ASD (245). Mice injected with tunicamycin buried a significantly higher percentage of marbles as compared to vehicle-treated or cysteamine pretreated mice ($p < 0.05$; Figure 13c), which indicates increased repetitive digging behavior. The tunicamycin treated mice also spent significantly increased time grooming than vehicle or cysteamine pretreated mice ($p < 0.05$; Figure 13d). No significant change in the number of bouts of grooming was observed in mice following tunicamycin treatment (Figure 13d), but cysteamine pretreatment reduced grooming bouts as compared to tunicamycin alone treated mice. This suggests that ER stress induces increased grooming time, but does not alter the time of each grooming bout, though cysteamine treatment alone may reduce grooming behavior.

Tunicamycin treated mice exhibited deficits in communication, as indicated by reduced number per minute and total duration of ultrasonic vocalizations (USV) produced in response to a social encounter which is prevented with cyateamine pretreatment ($p < 0.05$; Figure 13e).

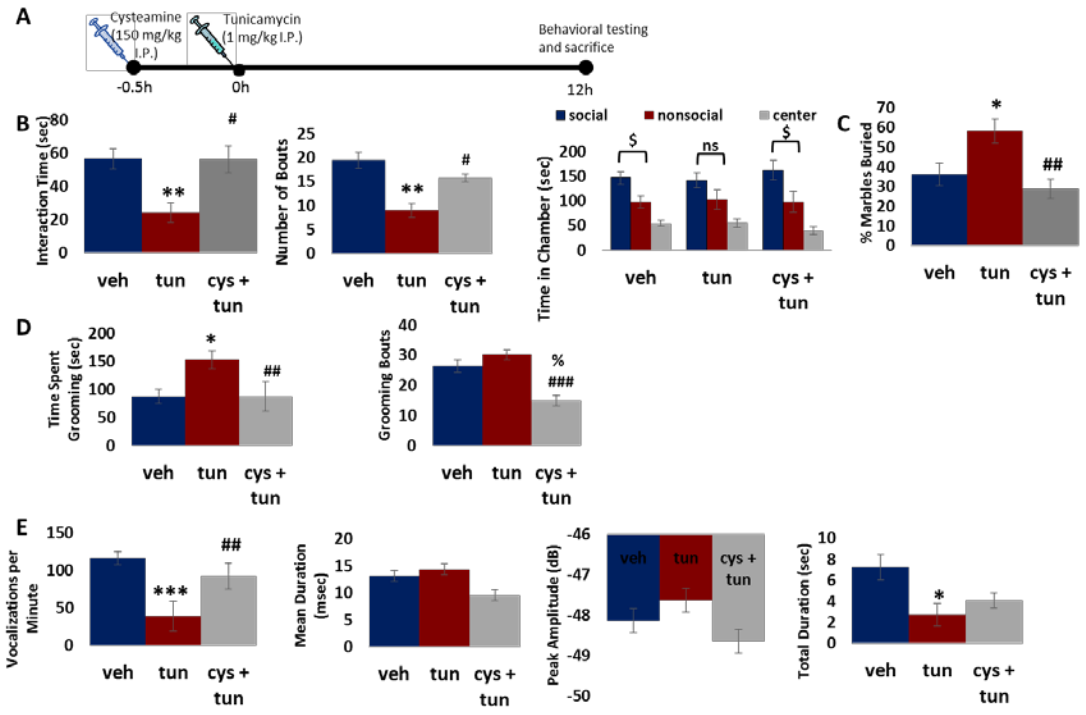

Figure 14. Cysteamine attenuates ER stress-induced ASD-like behavior in mice. A) Treatment paradigm. B) Reciprocal social interaction test, C) marble bury test, D) forced swim test, E) grooming test, and ultrasonic vocalization recordings (G-J) were performed on 3 month old male mice 12 hours after tunicamycin injection. *p<0.05 vs. veh, **p<0.01 vs. veh, ***p<0.0001 vs. veh, %p<0.001 vs. veh, # p<0.05 vs. tun, ##p<0.01 vs. tun, ###p<0.001 vs. tun, $p<0.0001 vs tun. tun = tunicamycin; veh = vehicle; cys = cysteamine.

To further determine the role of TG2 in ER stress-induced ASD-like behavior, we silenced TG2 expression in mouse PFC. Mice were injected with control siRNA (con siRNA) or transglutaminase 2 siRNA (TG siRNA) lentiviral particles, then allowed to recover for 2 weeks before being treated with tunicamycin or vehicle (Figure 14a). TG2 siRNA injected mice showed significantly lower levels of TG2 protein than con siRNA injected mice (Figure 14b). We found that tunicamycin induced ASD-like behavior as demonstrated by significant changes in social interaction time during the reciprocal social interaction test ($p < 0.01$; Figure 14c), marble bury test ($p < 0.05$; Figure 14d) and time spent grooming ($p < 0.05$; Figure 14e) in control siRNA-treated mice, but not in TG2 siRNA-injected mice. Grooming bouts were not significantly altered in by tunicamycin between control siRNA and TG2 siRNA groups ($p > 0.05$; Figure 14g). Together, these findings suggest that tunicamycin treatment induces ASD-like behavioral abnormalities in male mice and inhibition of TG2 through pharmacological or lentiviral manipulation prevents the development of behavioral deficits.

Transglutaminase 2 overexpression in neurons results in ASD-like Behavior in male mice.

To determine if TG2 is sufficient for inducing ASD-like behavioral deficits in mice, we compared WT mice and mice overexpressing TG2+/+. TG2+/+ mice interacted significantly less with a stranger mouse than wild-type littermates during the reciprocal social interaction test ($p<0.05$; Figure 15b). The three chamber test did not show alterations in chamber preference ($p<0.0001$; Figure 15b), though this test is not considered to be as sensitive as the reciprocal social interaction test and may not show

changes when other measures of social interaction and social preference will (243). Mice overexpressing TG2 also showed altered communication in the ultrasonic vocalization test (Figure 15d). TG2+/+ mice showed significantly fewer vocalizations per minute as well as total duration of vocalizations, but no alterations in mean peak amplitude and mean duration of calls. This suggests that TG2+/+ mice show alterations in overall amount of communication, but the types of calls are not changed. TG2+/+ mice did not show alterations in obsessive behaviors in the marble bury and grooming tests ($p>0.05$; Figure 15a and Figure 15c) as compared to WT. Together, these findings suggest that TG2 mediates abnormalities in the social aspects of ASD-like behavior in male mice, but not obsessive behaviors.

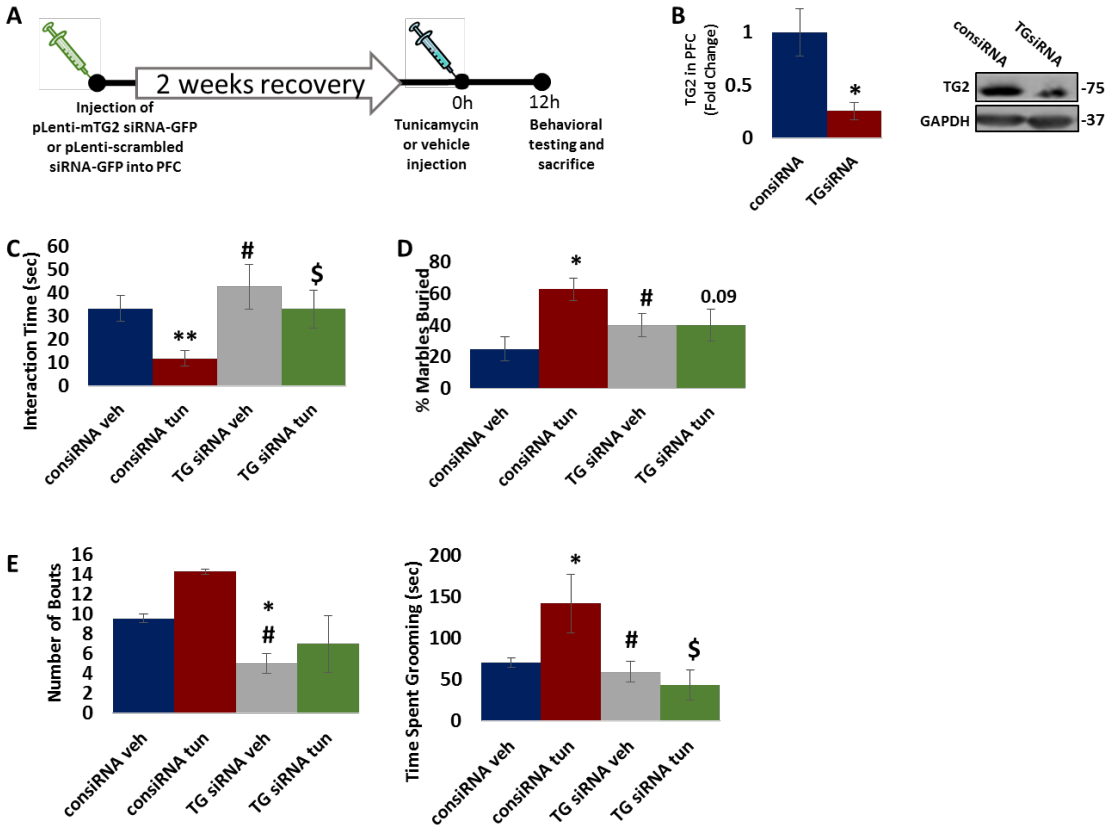

Figure 15. Knockdown of TG2 in PFC attenuates ER stress-induced ASD-like behavior in mice. A) Treatment paradigm. B) Photo image to show no damage to cortex at injection sites and western blotting to show decreased TG2 protein expression in PFC of TG2 siRNA-injected mice. C) Reciprocal social interaction test, D) marble bury test, and forced swim tests were performed 12 hours after tunicamycin injection. *p<0.05 vs veh, **p<0.01 vs. veh, #p<0.05 vs. tun, ##p<0.01 vs. tun.

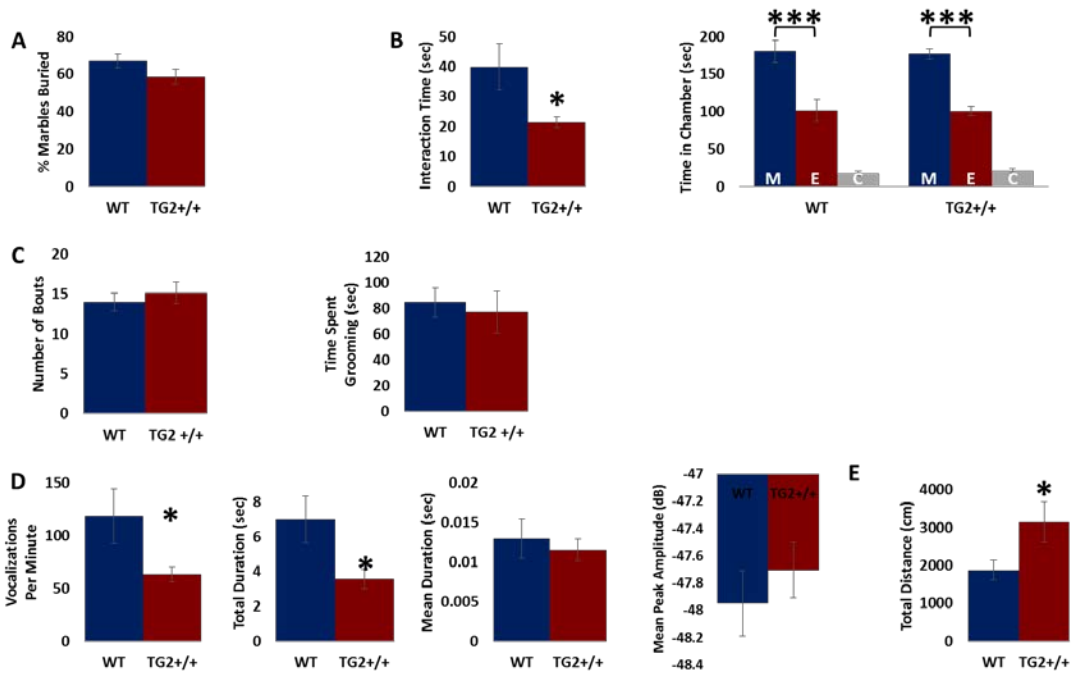

Figure 16. Transglutaminase 2 overexpression in neurons results in ASD-like Behavior in male mice. A) Social interaction test, B) marble bury test, D) grooming test, and E) open field test were performed on 3 month old male and female WT and TG2+/+ mice.

ERβ, but not ERα is altered in the frontal cortex of male and female WT and TG2+/+ mice.

Our previous studies show that ER stress induces alterations in ERβ in PFC (240), so we isolated the PFC from WT and TG2+/+ mice and performed western blotting to determine ERβ and ERα protein levels in males and females. TG2+/+ Males show reduced ERβ, but not ERα in PFC as compared to WT (Figure 16a and 16b). We found no changes in ERβ or ERα in female TG2+/+ mice as compared to WT (Figure 16c and 16d).

Treatment with ERB-041 reverses ASD-like behavior in male TG2+/+ mice.

In order to determine if boosting ERβ signaling can reverse the behavioral changes seen in male TG2+/+ mice, we treated these mice chronically for 2 weeks with an ERβ agonist, ERB-041 or vehicle. Behavioral tests were then performed according to the schematic diagram (Figure 17a). To test whether ERB-041 administration reverses obsessive and repetitive behaviors induced by TG2 overexpression, marble burying and grooming tests were performed. As was found in Figure 15, TG2 +/+ vehicle mice showed no alterations in marble bury (Figure 17b) and grooming behaviors ($p > 0.05$; Figure 17c) as compared to WT vehicle. ERB-041 significantly ($p< 0.05$ vs. WT veh and TG2 +/+ veh) reduced marble burying behaviors in both WT and TG2+/+ mice, suggesting that there ERB-041 has an effect on marble burying behavior regardless of genotype. TG2 +/+ vehicle treated mice exhibited deficits in vocalizations per minute and total duration ($p < 0.05$; Figure 17d) as compared to WT vehicle and WT ERB-041 treated groups, similar to the results in Figure 15. ERB-041 treatment reversed these

deficits in TG2 +/+ mice ($p<0.05$; Figure 17d). Together, these findings show that agonizing ERβ can reverse social interaction and communication deficits in male mice overexpressing TG2.

TG2 protein and mRNA levels are significantly increased in ASD subjects as compared to age- and sex-matched controls.

Human prefrontal cortical tissues from the middle frontal gyrus were used to determine if subjects with ASD show increased mRNA and protein levels of TG2 as compared to age and sex-matched controls. We found that ASD subjects show increased protein levels of TG2 as compared to controls ($p<0.05$; Figure 18a). We also found that ASD subjects show increased mRNA levels of TG2 as compared to controls ($p<0.05$; Figure 18b).

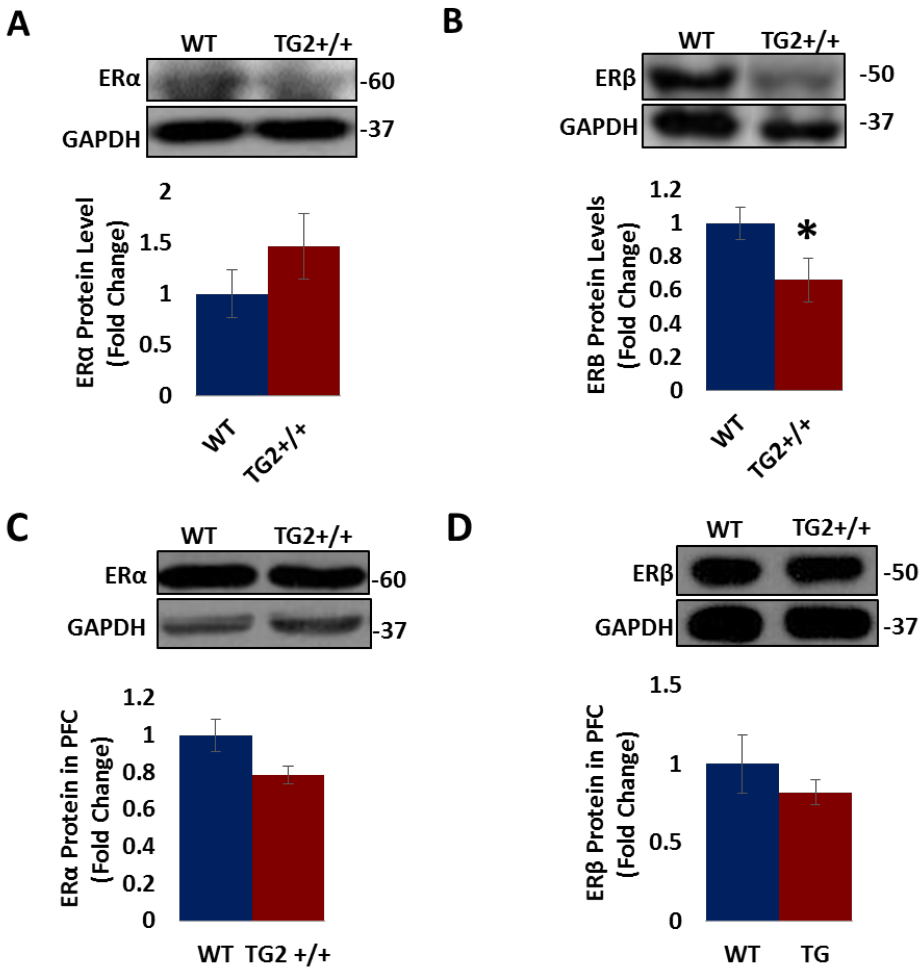

Figure 17. ERβ and ERα in the frontal cortex of male and female WT and TG2+/+ mice.

A) Representative blot and densitometric analysis of ERα in PFC of WT and TG2+/+ male mice. B) Representative blot and densitometric analysis of ERβ in PFC of WT and TG2+/+ male mice. C) ERα in female mice, D) ERβ in female mice. ERα and ERβ were normalized to GAPDH or tubulin as a control. *$p<0.05$

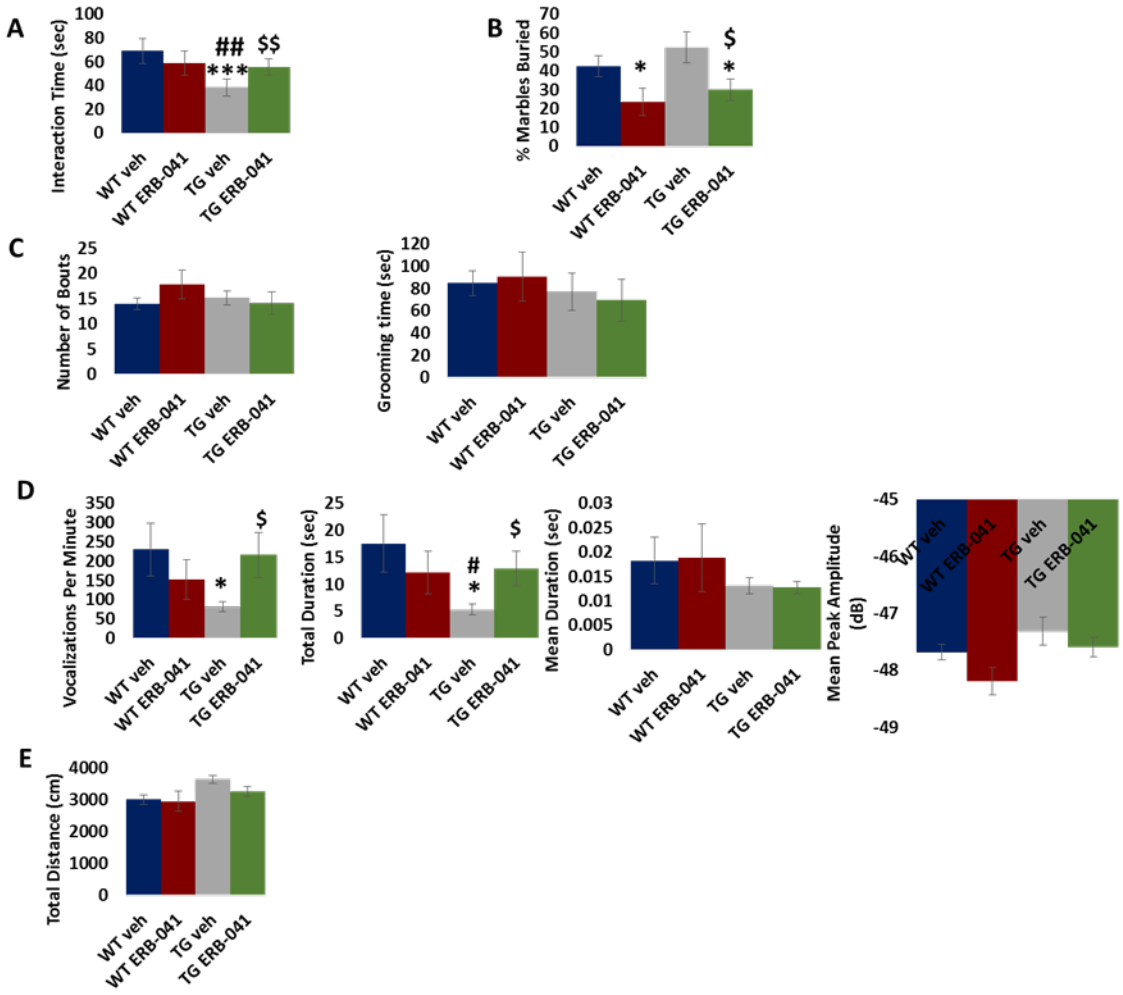

Figure 18. Treatment with ERB-041 reverses ASD-like behavior in male TG2+/+ mice.

A) Social interaction test, B) marble bury test, D) grooming test, and E) open field test were performed on 3 month old male mice.

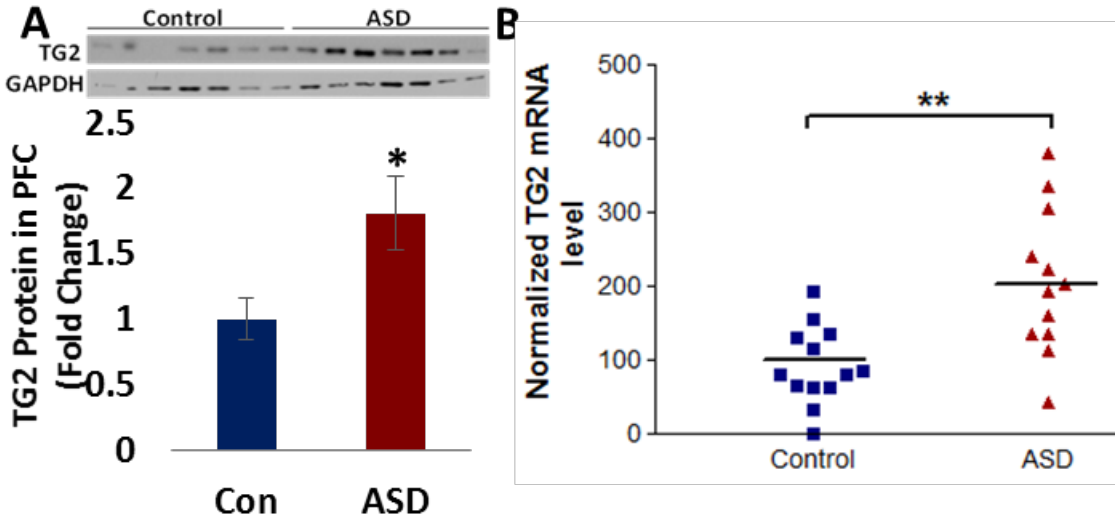

Figure 19. TG2 protein and mRNA levels are significantly increased in ASD subjects as compared to age- and sex-matched controls.

A) Normalized TG2 protein in medial frontal gyrus of human subjects. B) Normalized TG2 mRNA in medial frontal gyrus of human subjects.

Discussion

Emerging evidence suggests that ER stress may be one potential mechanism involved in synaptic deficits and behavioral alterations found in ASD (127, 187). At the molecular level, ER stress arising from genetic variations in several synaptic genes implicated in ASD (such as neuroligin, neurexin, CNTNAP and CADM1) has been shown to cause impaired synaptic function and signal transduction (127, 187). In the present study, we used the glycosylation inhibitor, tunicamycin to induce ER stress in mice to determine if induction of this cellular response results in behavioral alterations homologous to ASD. Tunicamycin has been used as an inducer of ER stress in various cellular systems (199; 200, 247). The ER contains the largest reservoir of calcium in the cell, and ER homoeostasis is maintained by Ca^{2+} uptake and efflux through various transporters and ion channels on the ER membrane that are controlled by intracellular signaling cascades (247-249). Depleting the ER Ca^{2+} store by inhibiting ER membrane Ca^{2+} pumps or through uncontrolled ER Ca^{2+} release results in ER stress and cell death (250)

TG2 is known to inhibit aromatase activity and expression (16). TG2 also has two Estrogen Response Elements (EREs) (14) and single nucleotide polymorphisms (SNPs) in the ERE binding region of TG2 can alter the sensitivity of TG2 to fluctuating estrogen levels (17). Our previous data has shown that ER stress induces ASD-like behavioral deficits and reduction in ERβ, which can be reversed by treatment with ERB-041, an estrogen receptor beta agonist (240). TG2 is a known component of ER stress and could be the downstream mediator of the ER stress-induced behavioral deficits. Global overexpression of TG2 results in a reduction in ERβ along with social interaction and

communication deficits, similar to treatment with tunicamycin. This shows that TG2 is sufficient to induce ASD-like social and communication deficits in mice. Knocking down TG2 in the PFC through lentiviral injection prevents deficits in social interaction and communication, suggesting that TG2 is necessary for the ER stress-induced behavioral deficits. Pharmacological manipulation of TG2 through pretreatment with cysteamine also prevents tunicamycin-induced ASD-like social and communication deficits, reiteration the necessity of TG2 for the ER stress-induced behavioral deficit response.

This study further explores the molecular pathways involved in social and communication deficits induced by the ER stress response and regulation of ERβ in ASD. Studies like this one continue to add to the growing body of literature involving ASD and estrogen signaling. ERB-041 and similar ERβ agonists are being tested for use in other disorders, but may provide a treatment option for individuals with ASD and other social or neurodevelopmental disorders. Further studies into possible downstream molecules of TG2 as well as regulation of TG2 and its role in social behavior developmentally are needed.

V. DISCUSSION

ASD is a neurodevelopmental disorder that is rapidly increasing in prevalence. It currently affects approximately 1 in 68 children in America and approximately 1% of the worldwide population (1). This disorder is complex, with each patient exhibiting slightly different manifestations of symptoms across the three major domains: social deficits, language/communication deficits, and repetitive behaviors. There are no treatments for ASD, though some drugs are used off-label to dampen individual symptoms of the disorder. These drugs have mixed efficacy and patients can show significant side effects that outweigh the benefits of the drugs. Behavioral therapy is the most effective option for individuals with ASD, though these interventions must begin early in life to have better outcomes of behavioral and symptom modification. Though it is difficult, studying ASD is extremely important in order to develop new and better treatments for patients suffering from this often debilitating disease as well as their families.

This thesis project has advanced the knowledge of the field on the role ER stress in social behavior, the role of ERβ in preventing the IRE1/XBP1 pathway induced by ER stress, and the role of an enzyme called transglutaminase 2 as the downstream mediator of ER stress in mice. Although ER stress had previously been implicated in ASD, the underlying mechanism(s) were not known.

To investigate this question, we developed a novel mouse model of social deficits by inducing ER stress through tunicamycin administration. This model offers an alternative to the Maternal Immune Activation (MIA) model to study social deficits in mice in a non-genetic based model. The ER stress model showed a gender-specific phenotype with males showing social deficits and reduced ERβ protein levels in the PFC and females showing no behavioral deficits and no change in ERβ. This is very similar to clinical manifestations of ASD, as it is 5 times more common in males than females. In addition, postmortem data in this study showed that individuals with ASD show reduced ERβ protein and mRNA levels, similar to the acute tunicamycin treated mice. In the tunicamycin treated mice, social behavior deficits, IRE1 phosphorylation, and CHOP mRNA levels were prevented by ERB-041 pretreatment before ER stress insult. ERB-041 is an estrogen receptor beta agonist, which has been administered to both men and women in clinical trials for rheumatoid arthritis with no reported sexual side effects (clinicaltrials.gov).

The effectiveness of this drug and lack of reported sexual side effects in men show that ERB-041 may be a candidate drug for treatment of ASD. However, the role of ERβ in the pathophysiology of ASD needs to be further studied. Phenotyping ERβ knockout mice for ASD-like behaviors including social deficits or knocking down ERβ in PFC using lentiviral particles may be helpful in determining the role of ERβ in the pathophysiology of ASD. Overexpression of ERβ in the PFC in order to determine if this can prevent ER stress-related behavioral deficits may be helpful in determining if ERβ is in fact an effective drug target in ASD.

In the human postmortem studies relating to estrogen signaling in ASD, it was clear that levels of cofactor mRNA transcripts SRC-1, CBP, and nCOR were correlated with higher (worse) scores in the ADI-R test, which determines ASD symptom severity. This study is novel, but the data was collected in a relatively small number of subjects. The alterations in the estrogen signaling system needs further investigation in larger sets of samples. Since brain tissue from individuals with ASD is quite scarce, lymphoblastoid cell lines that are banked for ASD cohorts (though there are several limitations including difference in tissue type and difficulties in transformation procedure) could provide a large sample of biological material to understand the pathophysiology of ASD. Studies like this one continue to add to the growing body of literature involving ASD and estrogen signaling. ERB-041 and similar ERβ agonists are being tested for use in other disorders, but may provide a treatment option for individuals with ASD and other social or neurodevelopmental disorders.

Transglutaminase 2 (TG2) was chosen as a possible downstream mediator of the ER stress-induced social deficits because of its previous implication in depression, a comorbidity of ASD (Pandya et al., 2016), its role in ER stress, and its proximity to other ASD susceptibility genes on chromosome 20q11.2. TG2 is known to be induced by ER stress and this study showed that it is increased in PFC of mice treated with tunicamycin. Significantly increased TG2 in the PFC can result in significant alterations in behavior as the PFC is important in learning, memory, and social behavior. The PFC is also very important in ASD as synaptic changes in this region can result in behavioral deficits seen

in the disorder. Knockdown of TG2 via lentiviral injection in the PFC prevents tunicamycin-induced social behavior deficits and obsessive behaviors. This shows that TG2 is necessary to induce ASD-like social and communication deficits in mice. Pharmacological manipulation of TG2 through pretreatment with cysteamine also prevents tunicamycin-induced ASD-like social and communication deficits, reiteration the necessity of TG2 for the ER stress-induced behavioral deficit response. Furthermore, transglutaminase 2 mRNA and protein levels were increased in the same human postmortem tissues showing reduced estrogen receptor beta expression (Figure 18). Higher TG2 mRNA levels were positively correlated with ADI-R scores for three domains of the disorder, suggesting that TG2 can alter cognitive function and result in increased symptoms seen in ASD. Cysteamine, a TG2 inhibitor, is an FDA-approved drug for treating cystinosis. This may provide another treatment option for individuals with ASD without risk of sexual side effects related to the estrogen system. The drug could be repurposed to treat social deficits and repetitive behaviors in individuals with ASD.

Though TG2 seems to be important for the development of ER stress-related behavioral deficits, its role still needs to be further studied. Overexpression of TG2 in mice shows mixed results, with these mice showing reduced social interaction in the reciprocal interaction test, but no change in chamber preference in the three chamber test. This is a puzzling result. Since TG2 is genetically overexpressed, these mice may have some developmental compensatory mechanism which dampens the effects of TG2 by the adult stage. Since these results are unexpected, further studies on overexpression of TG2

specifically in the PFC may provide a better understanding of the role of TG2 in ASD-like behavioral deficits. Additionally, TG2 is expressed ubiquitously, which means it is expressed in almost every tissue and cell type in the body. Knocking down TG2 in different cell types in the brain using the Cre-Flox system in mice may provide answers on which cell type is most important in the development of social and repetitive behavior deficits involving TG2. TG2 is more prominently expressed in excitatory CamK neurons in the brain, so knocking down TG2 in this cell type may prevent ER stress-induced behavioral deficits in mice.

The knowledge of environmental insults that result in ASD in humans is severely lacking. This study has elucidated the molecular signaling mechanisms that result in ER stress-induced social behavior deficits in mice and has shown that these signaling pathways are similarly altered in human postmortem tissues. The study provided some initial evidence on estrogen receptor β signaling as a potential therapeutic target in ASD. This study was crucial to understand ASD pathophysiology in humans and has paved the way for more studies in gender specificity and environmental causes of ASD. More work must be done in this area of study, but continuing to study ASD is necessary to reduce the burden of this disorder on patients, families, and society.

VI. SUMMARY

Autism Spectrum Disorders are more prevalent in boys than in girls, with ratios of 4.5:1, suggesting the possible role of sex hormones in the pathophysiology of this set of disorders. In addition to the extreme male brain theory on the high levels of testosterone during early development as a risk factor for ASD, a number of recent studies have shown the role of estrogens in the development of ASD. Molecular alterations within the estrogen receptor signaling pathway may contribute to the sex difference in ASD, but the extent of such abnormalities in the brain is not known.

Many studies have suggested an important role of ER stress in the pathophysiology of ASD. The ER is an important organelle responsible for the folding and sorting of proteins. Disturbances ER homeostasis can trigger a cellular response known as the unfolded protein response (UPR), leading to accumulation of unfolded or misfolded proteins in the ER lumen called ER stress. A number of recent studies suggest that mutations in ASD susceptible synaptic genes induce ER stress. Although a number of recent studies have suggested the role of ER stress in the pathophysiology of autism spectrum disorder (ASD) in humans, the underlying mechanism(s) is not known.

Studies have shown that ER stress induces the activity of TG2, a Ca2+ dependent enzyme involved in neural development and functioning (15, 16). TG2 has many functions, some

of which are transamidation, deamidation, histone modifications, and posttranslational modifications of proteins (23, 24) TG2 is located on the chromosome 20q11.2, a region associated with susceptibility to ASD (17). Moreover, a gene expression study has reported higher TG2 expression in the frontal cortex of ASD subjects (15). A significant increase in serum levels of IgA antibodies to TG2 has also been reported in children with ASD (4, 228)..

1. Dysregulation of estrogen receptor beta (ERbeta), aromatase (CYP19A1) and ER co---activators in the middle frontal gyrus of autism spectrum disorder subjects.

Postmortem middle frontal gyrus tissues (13 ASD and 13 control subjects) were used. The protein levels were examined by western blotting. The gene expression was determined by qRT-PCR. Gene expression analysis identified a 35% decrease in ERβ mRNA expression in the middle frontal gyrus of ASD subjects. In addition, a 38% reduction in aromatase (CYP19A1) mRNA expression was observed in ASD subjects. We also found significant decreases in ER co-activators that included a 34% decrease in SRC-1, a 77% decrease in CBP, and a 52% decrease in P/CAF mRNA levels in ASD subjects relative to controls. There were no differences in the mRNA levels of TIF-2, AIB-1 (ER co-activators), ER co-repressors (SMRT and nCoR) and ERα in the middle frontal gyrus of ASD subjects as compared to controls. We observed significant correlations between ERβ, CYP19A1, and co-activators in the study subjects.

Immunoblot analysis further confirmed the changes in ERβ and aromatase at the protein level in the control and ASD subjects. These results, for the first time, provide the evidence of the dysregulation of ERβ and co-factors in the brain of subjects with ASD.

2. Estrogen receptor β attenuates endoplasmic reticulum stress---induced autism spectrum disorder---like behavior through IRE---1/XBP1 pathway.

In this study, treatment with tunicamycin, an ER stress inducer enhanced the phosphorylation level of inositol-requiring ER-to-nucleus signal kinase 1 (IRE1) and increased X-box-binding protein 1 (XBP1) mRNA splicing activity in the mouse prefrontal cortex (PFC), whereas inhibition of IRE1/XBP1 pathway by either a viral particle or pharmacological approach attenuated social behavioral deficits caused by tunicamycin treatment. Reduced estrogen receptor beta (ERβ) protein levels were found in PFC of male mice following tunicamycin treatment. Pretreatment with an ERβ specific agonist, ERB-041 significantly attenuated tunicamycin-induced deficits in social behavior, and activation of IRE1/XBP1 pathway in mouse PFC. Also, ERB-041 inhibited tunicamycin-induced increases in functional connectivity between medial prefrontal cortex (mPFC) and dorsal or ventral hippocampus in male mice. Together, these results show that ERβ attenuates ER stress-induced deficits in social behavior through IRE-1/XBP1 pathway.

3. Altered expression of endoplasmic reticulum stress---related genes in the middle frontal cortex of subjects with autism spectrum disorder.

This study investigated the mRNA expression of ER stress-related genes (ATF4, ATF6, PERK, XBP1, sXBP1, CHOP and IRE1) in postmortem middle frontal gyrus of ASD and control subjects. RT-PCR analysis showed significant increases in the mRNA levels of ATF4, ATF6, PERK, XBP1, sXBP1, CHOP and IRE1 in the middle frontal gyrus of ASD subjects. In addition, we found a significant positive association of sXBP1 mRNA with the diagnostic score for abnormality of development in ASD subjects. These results, for the first time, provide the evidence of the dysregulation of ER stress genes in the brain of subjects with ASD.

4. Estrogen receptor beta protects against endoplasmic reticulum stress---induced increases in transglutaminase 2 and ASD---like behavior in mice.

Adult male mice were injected intraperitoneally with ER stress inducer, tunicamycin (1mg/kg) and subsequently examined in social interaction, marble burying, grooming, ultrasonic vocalization and open field tests. The role of transglutaminase 2 was examined

using TG2 overexpressed mice, lentiviral and well as pharmacological approaches. Human postmortem samples included middle frontal gyrus samples from ASD subjects (n = 13) and age and sex-matched controls (n = 12). Tunicamycin treatment induced deficits in social behavior, increase in repetitive behavior and communication deficits. We found significant increase in TG2 mRNA and protein levels in mouse PFC following ER stress. Mice overexpressing TG2 in neurons showed ASD-like behavior. Moreover, TG2 overexpression in mouse PFC using lentiviral particles induced ASD-like behavior. Inhibition of TG2 using cysteamine or TG2 lentiviral particles in mouse PFC attenuated ER stress-induced ASD-like behavior in mice. Reduced estrogen receptor beta (ERβ), but not estrogen receptor alpha (ERα) protein levels were found in PFC of male mice overexpressing TG2. Moreover, ER beta agonist could significantly attenuate ER stress-induced ASD-like behavior in mice. Increased mRNA and protein levels of TG2 were also found in the middle frontal gyrus of ASD subjects as compared to controls.

VII. REFERENCES

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APPENDIX A: Supplemental materials from manuscript entitled "Dysregulation of estrogen receptor beta(ERbeta), aromatase (CYP19A1) and ER co-activators in the middle frontal gyrus of autism spectrum disorder subjects"

Table S1. List of primers used in the qRT-PCR.

Gene	Primer sequence (5' to 3')
CYP19A1-RP	GAT TTT AAC CAC GAT AGC ACT TTC G
CYP19A1-FP	CCC TTC TGC GTC GTG TCA T
SMRT-RP	CGG AAT CTT CCC CTC CTC CC
SMRT-FP	TGT GGT TCA TAA GCC ATC TGC
N-CoR-RP	TTG GAC TCT TGG ATG TGC C
N-CoR-FP	GCT GAT GAG GAT GTG GAT GG
P/CAF-RP	TGC CTC AAG TCC AGA AGA GG
P/CAF-FP	AGA ACA TTG CTT CGC TCG G
CBP-RP	TGT TGA ACA TGA GCC AGA CG
CBP-FP	TCA GTC AAC ATC TCC TTC GC
TIF2-RP	TCT GTG TAT GTG CCA TTC GG
TIF2-FP	TAA TGC ACA GAT GCT GGC C
SRC-1-RP	ACT ACT TGT CAT GCC AAC GG
SRC-1-FP	GCT CGT TCA TCC ACA TTG CC
AIB1-FP	TCA TAG GTT CCA TTC TGC CG
AIB1-RP	CGT CCT CCA TAT AAC CGA GC
ER-β-RP	CGT AAC ACT TCC GAA GTC GG
ER- β -FP	TCA CAT CTG TAT GCG GAA CC
ER-α-RP	GCC AGG CAC ATT CTA GAA GG
ER- α -FP	AGA CAT GAG AGC TGC CAA CC
GAPDH-FP	GAG TCA ACG GAT TTG GTC GT
GAPDH-RP	TTG ATT TTG GAG GGA TCT CG
Actin-FP	GGA CTT CGA GCA AGA GAT GG
Actin-RP	AGC ACT GTG TTG GCG TAC AG

Table S2. Correlations of mRNA transcripts with confounding variables.

	Age	PMI	Storage Time	pH	RIN
nCoR	0.174	-0.053	0.520*	-0.071	0.165
SMRT	0.107	-0.325	0.548*	-0.161	-0.022
CBP	0.046	-0.278	0.406*	-0.340	-0.390
P/CAF	-0.026	-0.313	0.440*	-0.403*	-0.153
TIF2	-0.251	0.042	0.439*	-0.311	-0.119
SRC1	-0.028	-0.278	0.462*	-0.379	-0.169
AIB1	-0.042	-0.231	-0.169	-0.262	-0.115
Erα	-0.183	0.182	0.260	0.146	0.074
Erβ	-0.254	-0.041	0.298	-0.090	-0.211
CYP19A1	-0.216	-0.127	0.196	-0.175	-0.297

Values are Pearson Correlation. PMI = Postmortem Interval; RIN = RNA integrity; *p < 0.05

Table S3. **Correlations between mRNA transcripts.**

Values are Pearson Correlation; *p <0.05.

	nCOR	**SMRT**	**CBP**	**P/CAF**	**TIF2**	**SRC1**	**AIB1**	**ERα**	**ERβ**	**CYP19A1**
nCOR	1	0.738* **0**	0.199 0.364	0.192 0.38	0.34 0.112	0.496* **0.019**	0.047 0.825	0.084 0.703	0.042 0.849	0.055 0.809
SMRT	0.738* **0**	1	0.467* **0.025**	0.301 0.163	0.279 0.197	0.557* **0.007**	-0.035 0.869	0.306 0.155	0.221 0.31	0.141 0.533
CBP	0.199 0.364	0.467* **0.025**	1	0.818* **0**	0.483* **0.014**	0.787* **0**	-0.310 0.131	0.26 0.22	0.665* **0**	0.589* **0.003**
P/CAF	0.192 0.38	0.301 0.163	0.818* **0**	1	0.606* **0.001**	.800* **0**	-0.211 0.309	0.221 0.3	0.568* **0.004**	0.631* **0.001**
TIF2	0.34 0.112	0.279 0.197	.483* **0.014**	.606* **0.001**	1	0.651* **0.001**	-0.104 0.618	0.268 0.205	0.376 **0.07**	0.319 0.138
SRC1	0.496* **0.019**	0.557* **0.007**	0.787* **0**	0.800* **0**	0.651* **0.001**	1	-0.075 0.725	0.109 0.614	0.456* **0.025**	0.443* **0.034**
AIB1	0.047 0.825	-0.035 0.869	-0.310 0.131	-0.211 0.309	-0.104 0.618	-0.075 0.725	1	-0.076 0.719	-0.042 0.843	0.167 0.435
ERα	0.084 0.703	0.306 0.155	0.26 0.22	0.221 0.3	0.268 0.205	0.109 0.614	-0.076 0.719	1	0.752* **0**	0.503* **0.012**
ERβ	0.042 0.849	0.221 0.31	0.665* **0**	0.568* **0.004**	0.376 0.07	0.456* **0.025**	-0.042 0.843	0.752* **0**	1	0.885* **0**
CYP19A1	0.055 0.809	0.141 0.533	0.589* **0.003**	0.631* **0.001**	0.319 0.138	0.443* **0.034**	0.167 0.435	0.503* **0.012**	0.885* **0**	1

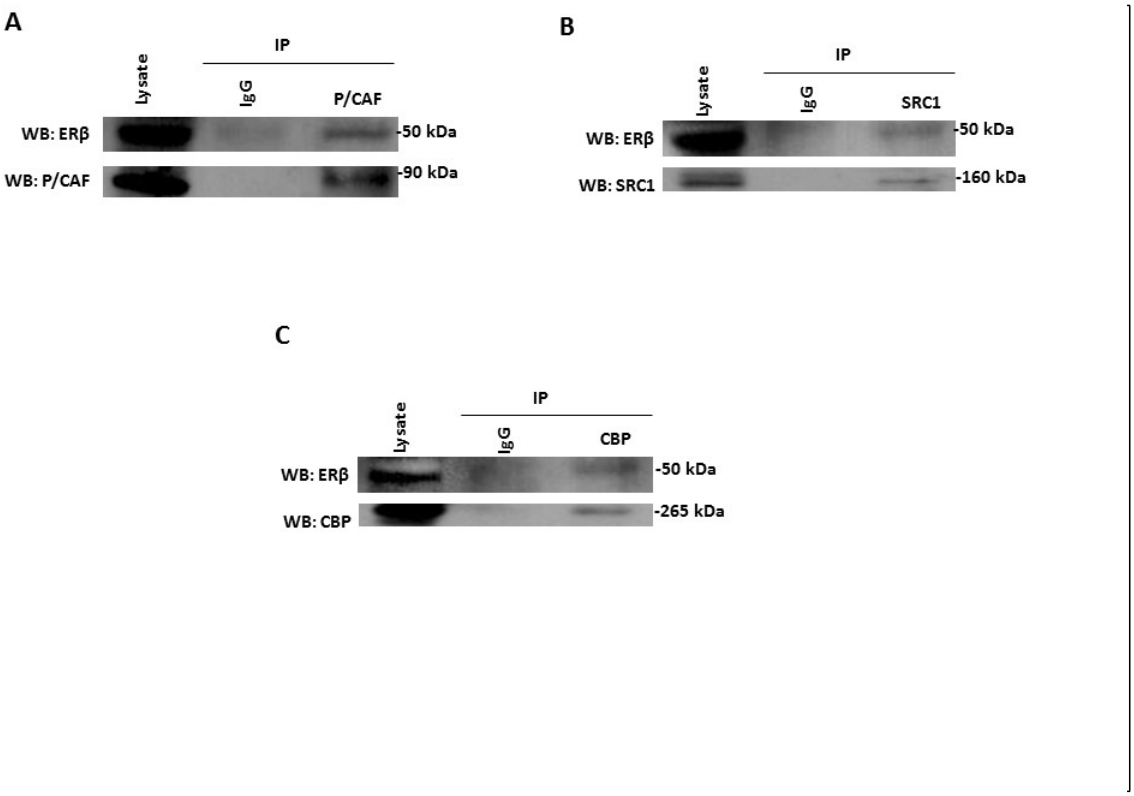

Figure S1: ERβ is associated with the co-factors, P/CAF, SRC1, or CBP.

Lysates from postmortem middle frontal gyrus of control subjects were subjected to immunoprecipitation (IP) using a co-factor antibody followed by western blotting (WB) with the ERβ or co-factor antibody. A separate IP assay was performed for (A) P/CAF, (B) SRC1, or (C) CBP. Lysate represents 10% of the amount used in the IP. IgG, IgG control.

APPENDIX B: Supplemental materials from manuscript entitled "Altered expression of endoplasmic reticulum stress-related genes in the middle frontal cortex of subjects with autism spectrum disorder"

Table S4. Primer sequences

Species	Gene	Forward Sequence	Reverse Sequence
Human	ATF4	5'- GCTAAGGCGGGCTCC TCCGA -3'	5'- ACCCAACAGGGCATCCAA GTCG -3'
Human	ATF6	5'- ATGAAGTTGTGTCAG AGAACC -3'	5'- CTCTTTAGCAGAAAATCC TAG -3'
Human	PERK	5'- AATGCCTGGGACGTG GTG GC -3'	5'- TGGTGGTGCTTCGAGCCAGG -3'
Human	XBP1	5'- GGCATCCTGGCTTGCCTC CA -3'	5'- GCCCCCTCAGCAGGTGTTCC -3'
Human	sXBP1	5'- CGCTTGGGGATGGAT GCCCTG -3'	5'- CCTGCACCTGCTGCGGACT -3'
Human	CHOP	5'- GGAGCATCAGTCCCC CACTT -3'	5'- TGTGGGATTGAGGGTCAC ATC -3'
Human	IRE1	5'- TGCTTAAGGACATGGCTA CCATCA -3'	5'- CTGGAACTGCTGGTGCTGGA -3'
Human	18S	5'- GGCCCTGTAATTGGAATG AGT C -3'	5'- CCAAGATCCAACTACGAG CTT -3'

ATF4 =Activating Transcription Factor 4; ATF6 = Activating Transcription Factor 6; PERK = Protein Kinase-like Endoplasmic Reticulum Kinase; XBPI = X-box Protein I; sXBPI = Spliced X-Box-Protein 1; CHOP = CCAAT-enhancer-binding protein homologous protein; IRE1 = Inositol Requiring Enzyme 1