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Unfolded protein response and associated alterations in toll-like receptor expression and interaction in the hippocampus of restraint rats

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Abstract

Recent evidence suggests that the cellular response to stress often elicits the unfolded protein response (UPR), which has an active role in major depression in emotionally relevant regions of the brain, such as the hippocampus. Much of the UPR activity has been found to be coalesced with the pro-inflammatory environment of the depressed brain. Specifically, downstream transcriptions of pro-inflammatory cytokines and increased regulation of candidate inflammatory mediators, such as toll-like receptors (TLRs), are promoted by the UPR. The present study examined the hippocampus associated expression profile of *Tlrs* genes and their interaction with the UPR chaperone GRP94 in stress-induced rodent model of depression (restraint stress model). Also, expression status of UPR related genes were evaluated in hippocampus using the same model. mRNA and protein levels of *Tlrs* and UPR associated genes were examined by qRT-PCR and Western blot, respectively. Co-immunoprecipitation (Co-IP) method was used to determine the direct interaction between TLRs with GRP94 in depressed rat brain. The results showed that both UPR (*Xbp-1,* its spliced variant *sXbp-1*, *Atf-6*, *Chop*, and *Grp94*) and *Tlr* (2, 3, 4, 7 and 9) genes were significantly upregulated in the hippocampi of rats who were exposed to restraint stress. Similar upregulation was observed in the protein levels of the above-mentioned TLRs and the UPR chaperone protein GRP94 as well as total and phosphorylated forms of sensor proteins IRE1α and PERK. Further, a significantly increased interaction was observed between GRP94 and the activated TLR proteins. Since, increased inflammatory activity in vulnerable areas like

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hippocampus is coherently associated with depressed brain; our present data suggest that the UPR may be an integral part of increased activity of inflammatory regulations in depression.

Keywords

Restraint stress; rat; depression; UPR; TLR; hippocampus

1. INTRODUCTION

MDD is a psychiatric disorder that affects as much as 10% of the population with a lifetime incidence of 12% in men and 20% in women. Hallmarks of the disease include feelings of sadness, guilt, lethargy, changes in sleeping and eating patterns as well as subsequent weight gain or loss (Belmaker and Agam, 2008). Contributing factors of the depressive phenotype are thought to be environmental, genetic, and epigenetic in nature (Lopizzo et al., 2015); however underlying pathophysiological mechanisms associated with depression are still not clearly understood.

The unfolded protein response (UPR) can generally be described as the cellular response to environmental stressors (Khan and Schröder, 2008). Such stressors cause abnormalities in the quality of translated proteins in the endoplasmic reticulum. Consequently, the changes associated with misfolded proteins trigger the activation of three branches of the UPR first by dissociation of the chaperone protein GRP78 and then by subsequent release of activating transcription factor 6 (ATF6), oligomerization of inositol-requiring protein-1α (IRE1α), and/or the dimerization of protein kinase RNA-like endoplasmic reticulum kinase (PERK). The extent and duration of the stress determines which of these branches will become active. For example, acute stress may have ATF6 released with oligomerization of the IRE1α sensors, while a prolonged and extremely stressful environment would promote the activity of PERK and the C/EBP-homologous protein (CHOP)-mediated signaling pathway. These events initiate downstream signaling cascades which ultimately act on transcription, translation, and activation or repression of genes that can lead to inflammation, and even apoptosis (Kaufman, 2002; Ron, 2002).

We and other investigators have shown that the activity of UPR system may be responsible for some of the underlying pathophysiology of stress related disorders such as depression and have suggested that this response may intimately be involved in downstream pathways such as apoptosis, inflammation and dysfunctional cellular communication (Abrous et al., 2005; Calvano et al., 2005; Timberlake and Dwivedi, 2016). In the learned helpless model, which is a well-established rodent model of stress-induced behavioral depression (Henn and Vollmayr, 2005; Maier, 1984), we found that gene expression from each of the three branches of the UPR were upregulated in the hippocampus (Timberlake and Dwivedi, 2016); the three branches of activity being the events downstream of the activation of the sensors ATF6, IRE1α, and PERK (Wang and Kaufman, 2014). These data are consistent with the previous preclinical studies in hippocampus of restraint rats (Zhang et al., 2014) and clinical studies in temporal cortex of MDD brain (Bown et al., 2000), which showed expression

related changes of UPR chaperones GRP78 and GRP94 as well as hippocampal shrinkage (Bremner et al., 2000) and hippocampal apoptosis (Zhang et al., 2014).

As mentioned earlier, changes in inflammation are intimately linked to depression (Miller and Raison, 2016; Raison et al., 2006). When in a state of cellular stress, one of the first responses of the UPR is to increase the recruitment of chaperones to the membrane that are responsible for folding, or aiding in the folding of proteins. Previously, we showed that *Grp78* and *Grp94* were significantly upregulated at the transcriptional level in hippocampus of learned helpless rats (Timberlake and Dwivedi, 2016). GRP94, specifically, is important in folding toll-like-receptors (TLRs) which are closely involved in mediating the inflammatory response (Garg et al., 2012; Raison et al., 2006; Staron et al., 2010; Takeda and Akira, 2005; Yang et al., 2007; Zacharowski et al., 2006). In the context of depression, TLRs 2–5, 7 and 9 have been shown to be upregulated in peripheral blood or brain of depressed individuals (Hung et al., 2014; Pandey et al., 2014). Interestingly, these TLRs are folded by GRP94 except TLRs 3 and 5 (Yang et al., 2007). Downstream of these phenomena can lead to production of pro-inflammatory cytokines which have been shown to be elevated in depression (Takeda and Akira, 2005). How altered UPR system is linked to inflammatory changes in depressed brain, however, is not clearly understood.

The purpose of the present study, therefore, is several folds: 1) to confirm our previous finding of altered UPR in depression using a different animal model of depression (chronic restraint); 2) whether TLRs are altered in depressed brains; and 3) whether altered expression of TLRs are associated with a specific chaperone molecule of the UPR system. In this study we have used the chronic restraint model as it is well established model of stress and has shown to impact both behavior (like the forced swim test and EPM) and physiology (changes in body weight, food intake, and water intake as well as sucrose preference) of rats that undergo the stress paradigms (Chiba et al., 2012; Klenerová V et al., 2007; Torres et al., 2002; Xueer Wang et al., 2016). More specifically, we examined the expressions of *Grp94*, *Xbp-1* (including transcript variant s*Xbp-1*), *Atf6*, *Atf4*, and *Chop* of the UPR pathway and *Tlrs* 2, 3, 4, 7 and 9 in the hippocampus of restraint rats, all of which have implications in depression (Bown et al., 2000; Green et al., 2008; Hung et al., 2014; Timberlake and Dwivedi, 2016; Zhang et al., 2014). We also examined the expression of GRP94 as well as TLRs 2, 4, 7 and 9 at translational levels. As part of activated UPR system we tested the expression levels of total (T) and phosphorylated (P) forms of both IRE-1 and PERK proteins. In addition, we studied the *in-vivo* interaction of the TLRs with GRP94 to show the relationship between the UPR and inflammatory system in the context of depression.

2. MATERIALS AND METHODS

2.1 Animals

Adult male Sprague-Dawley rats (250–300g body weight) were obtained from Envigo (Indianapolis, IN, USA) and housed in similar cages (2 rats/cage) within the same room under standard laboratory conditions (temperature 21 ±1°C, humidity 55 ±5%). Animals were given free access to food and water and adapted to the laboratory environment for 1 week prior to the experiment. Rats were randomly assigned to a naïve control group and chronic restraint stress group. Restraint stress was given to rats during the light cycle (08:00

to 12:00). All the experiments were carried out according to the National Institutes of Health (NIH) guide for the care and use of Laboratory animals and were approved by the Animal Care Committee (IACUC) of the University of Alabama at Birmingham.

2.2 Chronic restraint stress

Rats were placed individually in clear acrylic tubes (20 cm long, 6.35 cm internal diameter, air vents in the cap and along the tube) with the tail extending from the rear of the tube. The cap was placed inward enough to prevent the rat from moving forward or backward inside the tube. Rats were restrained for 2h/day for 14 consecutive days. Control rats were handled daily but not were restrained. All the studies were done in 7–9 controls and 7–9 restraint rats.

2.3 Tissue collection

Twenty-four hours after the final restraint session, rats were decapitated and brains removed. The brains were immediately flash frozen in liquid nitrogen and stored at −80°C. Brains were sectioned using a cryostat (Leica CM 1950) set at −20°C. The samples were suspended in a 1:1 mix of optimal cutting temperature compound OCT (Tissue-Tek; Sakura Finetek USA) and 30% sucrose solution (50 mL NaPO4 [1000 nm], 150 mL 3% saline, 300 mL 50% sucrose) and sliced into 300 μm slices. Whole-hippocampal tissue was dissected from brain slices. Hippocampal and remaining brain tissue samples were returned to −80°C storage until analysis.

2.4 RNA isolation

RNA was isolated using TRIzol® (Life Technologies, USA) as described earlier (Timberlake and Dwivedi, 2016). Initially the RNA samples were screened based on their purity (260/280 nucleic acid quantification; cut-off ≥1.8) as determined with NanoDrop spectrophotometer (ThermoScientific, Waltham, MA, USA). Afterwards, the RNA quality was further assessed using denaturing agarose gel electrophoresis and evaluating the 28S and 18S rRNA band integrity. Finally, only those samples were selected for analyses which showed 260/280 ≥1.8 and 28S:18S rRNA = 2:1.

2.5 QPCR based gene expression assay

M-MLV based reverse transcription of hippocampal RNA was performed following oligo (dT) priming method. One microgram (1 ug) total RNA was reverse transcribed using M-MLV Reverse Transcriptase (Invitrogen, Grandsland, NY, USA) and oligo $(dT)_{18}$ primer (Invitrogen, Grandsland, NY, USA). The oligo $(dT)_{18}$ primer annealing step was carried out at 5 μM concentration in presence of 1 mM dNTPs (Invitrogen, Grandsland, NY, USA) by incubating the reaction at 65°C for 5 min. The reaction was quenched by holding at 4°C for 2 min. The reaction was mixed with 1X first strand synthesis buffer (Invitrogen, Grandsland, NY, USA), 0.01 mM DTT (Invitrogen, Grandsland, NY, USA), 2 U of RNaseOut (Invitrogen, Grandsland, NY, USA) and 200 U of M-MLV Reverse Transcriptase and incubated at 37°C for 50 min. Finally, the reaction was inactivated at 70°C.

Relative abundance of transcripts was measured with a quantitative real time PCR machine (AriaMx Real-Time PCR System; Agilent Technologies, USA) using 1X EvaGreen qPCR

mastermix (Applied Biological Material Inc., Canada) in combination with 0.8 μM each of gene specific forward and reverse primers (Table 1). Forty-fold diluted raw cDNA was used as template for qPCR amplification using a thermal parameter of initial denaturation at 95°C for 10 min followed by a repeating 40 cycles of denaturation at 95°C for 10s, primer annealing at 60°C for 15s and an extension of amplicon at 72°C for 20s. Possibility of primer dimer formation and secondary product amplification was ruled out by running a single cycle of EvaGreen specific dissociation curve analysis program with initial denaturation at 95°C for 1 min followed by annealing at 55°C for 30s and repeat denaturation 95°C for 30s. Relative gene expression level was quantified after normalization with *Gapdh* as reference gene and fold change value was determined following Livak's $\Delta\Delta C$t calculation method (Livak and Schmittgen, 2001). Data are presented as fold change.

2.6 Western blot based protein quantification

Protein lysates prepared in RIPA buffer (Tris-Cl [pH 8.0] 50mM, NaCl 150 mM, NP-40 1%, Sodium Deoxycholate 1%, SDS 0.1%, supplemented with 1X complete protease inhibitor, 1mM of PMSF [phenylmethylsulfonyl fluoride] and 25μm of MG-132) were subjected to immunoblot analysis after resolving on denatured discontinuous SDS–polyacrylamide gel electrophoresis (SDS-PAGE). Probing with primary antibody was performed after titrating the optimum dilution point for detection of specific protein on blot (**Supplementary Table 1**). All the antibodies were diluted in 2% non-fat milk in TBST (1L of 1x tris-buffered saline and 1mL of Tween 20 [polysorbate 20]). β-actin was used to normalize the protein expression. Image J (v.1.51; https://imagej.nih.gov/ij/download.html) based densitometry quantification was performed by averaging at least three independent experiments. Fresh lysate was taken from the same tissue for a second batch of experiments which confirmed the findings of our previous observation with the first batch.

2.7 Co-IP based protein-protein interaction assay

Co-IP assay was performed as described previously (Dwivedi et al., 2009) with slight modifications. Briefly, tissue homogenate was prepared using mild NP-40 (Nonidet P-40) buffer and subjected to immunoprecipitation after removing the insoluble material by centrifugation. For affinity enrichment, supernatants were incubated with antibodies at 4°C overnight. Required amount of Protein A/G agarose beads was added to each antibody conjugated sample and were incubated at 4°C for 2 hrs for bead mediated immunoprecipitation. Subsequently, beads were washed three times with 500 μl of cold lysis buffer. Immunoprecipitates were finally eluted from the beads by adding 30 μl of SDS-PAGE sample buffer and heating for 20 min at 75°C. To eliminate the possibility of detecting non-specific interaction, a normal rat IgG antibody (Santa Cruz; SC-2026) was used throughout all Co-IP experiments which served as a negative control. The eluates were analyzed by western blotting following SDS-PAGE (Dwivedi et al., 2009).

2.8 Statistical analysis

Statistical Package for the Social Sciences (SPSS) was used for all the data analysis. The data are represented as mean ± SEM. Control and restraint groups were compared using independent sample t-test. Significance level was set at $p \leq 0.05$.

3. RESULTS

3.1 Body weight for rats

Rats were weighed on days 0, 7 and 14. The average weight for day 0 showed no significant difference between groups (Control: 310.67 ± 0.803; Restraint: 304.42 ±13.48; SEM = 1.123; p = 0.205). On day 7, there was a significant difference in weights between control and restraint (Control: 332.08 ± 0.927; Restraint: 299.42 ± 1.079; p <.001). On day 14, there was also a significant difference in weight gain between control and restraint rats (Control: 348 ± 0.974; Restraint 306.75 ± 1.187; p < 0.001). Overall, the control rats gained an average of 37.33 g over the two-week period while the restraint rats gained an average of 2.33 g; this data was statistically significant (p < 0.001).

3.2 mRNA expression analysis

Expression levels of *Grp94*, *Xbp-1*, *Atf6*, *Atf4*, and *Chop* associated with all three arms of the UPR as well relevant TLRs were examined in hippocampus of control and restraint rats. The expression levels of housekeeping gene *Gapdh* was used to normalize UPR genes which did not differ significantly between the control and restraint groups (p = 0.084). Similarly, for the TLR gene expression normalization, the *Gapdh* levels also did not differ significantly between the control and restraint groups (p = 0.085). The raw Ct values related to Gapdh expression is enlisted in **Supplementary Table 2.**

The expression levels of UPR genes are presented in Figure 1. As can be seen, *Xbp-1* was significantly upregulated in the restraint group compared with control group. The restraint group had 17.79% increase (p = 0.047). *Atf6* was significantly upregulated in the restraint compared with control rats having a 29.86% increase (p = 0.009). *Atf4* was also upregulated in restraint rats, however, this increase was not statistically significant (8.6% increase; p = 0.195). The downstream target of *Atf4, Chop*, on the other hand, was significantly upregulated in the restraint group (60.74% increase; p = 0.027). The chaperon molecule encoding gene *Grp94* was also significantly upregulated in the restraint rats with a 72.73% increase in its expression (p = 0.011). Furthermore, we show the expression level of *sXbp-1* transcript variant (*sXbp-1*) produced due to the splicing event between exon 4 and 5 of the *Xbp-1 gene*. Figure 2A shows the *Xbp-1* transcript structure in reference to exon 4 and 5 and the designing strategy of corresponding primer pair which amplify the specific *Xbp-1* splice variant (*sXbp-1*). Bar diagram in Figure 2B show that expression of *sXbp-1* transcript is increased by 42% in restraint rats compared to control rats (p = 0.039).

Figure 3 shows the expression levels of *Tlr* genes in hippocampus of restraint and control rats. It was observed that expression levels of *Tlr2* (p = 0.026), *Tlr3* (p = 0.049), and *Tlr7* (p = 0.032) were significantly upregulated in the range of 43–56% in the restraint rats compared with control rats. The expression of *Tlr4* was increased in restraint group by ~100% which was statistically significant (p = 0.047). The most dramatic change was observed in the expression of *Tlr9*, which was significantly upregulated in the restraint rats by 158% (p = 0.002).

3.3 Protein expression analysis

Western blot analysis was used to determine the expression changes in in various proteins. As can be seen in Figure 4, the expression of GRP94 was significantly upregulated in restraint rats compared with control rats with a 0.45 fold increase ($p = 0.031$). A 0.80 fold increase ($p < 0.01$) in the expression of IRE1-T, a 1.04 ($p = 0.029$) fold increase in the expression of IRE1-P, a 1.59 fold increase ($p < 0.001$) in the expression of PERK-T, and a 1.44 fold increase ($p = 0.06$) in the expression of PERK-P were observed in hippocampus of restraint rats. As far as TLRs were concerned, it was observed all the measured TLRs were upregulated in hippocampus of restraint rats compared with control rats (Figure 5). Individually, TLR2 was upregulated by 0.7-fold ($p = 0.012$); TLR4 by 0.5-fold ($p = 0.05$); TLR7 by 2.6-fold ($p = 0.06$); and TLR9 by 0.63-fold ($p < 0.001$).

3.4 Co-immunoprecipitation studies

Figure 6 shows the interaction results of GRP94 and TLRs as determined by co-immunoprecipitation using specific antibodies. The Co-IP assays of two controls and two restraint rats were shown as representative blots. The analyses of the blots showed that TLRs 2, 4, 7, and 9 had significantly increased interactions with GRP94 in hippocampus of restraint rats compared with control rats (TLR2: $p < 0.001$; TLR4: $p = 0.049$; TLR7: $p < 0.001$; TLR9: $p = 0.008$; $n = 4$/group). The fold increase for these interactions were 6.2, 5.52, 4.54, and 2.79, respectively for TLRs 2, 4, 7, and 9. As shown in the Western blot images, the negative control (IgG antibody) was not able to produce a significantly detectable signal due to non-specific interaction in any of the groups.

4. DISCUSSION

Biological or psychological stressors contribute to or initiate pathophysiology that underlies psychiatric illnesses that can impact learning and memory as well as emotional dysregulation. This is evident from both preclinical (Dwivedi, 2009; Nestler et al., 2002; Zhang et al., 2014) and clinical studies (Tollenaar et al., 2008). In this regard, the UPR system has been suggested as a possible agitator or even regulator of the underlying pathophysiology associated with major depression (Timberlake and Dwivedi, 2016). Our group previously reported that the genes (*Xbp-1*, *Atf-6*, *Chop*, and *Grp94*) of all three branches of the UPR (IRE1α, PERK, ATF6) were upregulated in hippocampus of learned helpless rats, a model of stress-induced depression (Timberlake and Dwivedi, 2016). The present study confirms these findings in a different model of depression, i.e., restraint-stress, where similar changes were noted in hippocampus of these rats, although the degree of changes were much greater in the restraint rats compared with learned helpless rats. This could be due to the type and amount of stress being applied to rodents. Procedurally, learned helplessness was induced with random mild tail shocks, whereas, restraint stress (2hr/day) was applied consecutively for 14 days. Restraint rats have been shown to have anxiety and depression-like behaviors under varying amounts of time and restraint paradigms (Chiba et al., 2012; Guedri et al., 2017; Zhang et al., 2014); in our case we followed a similar restraint paradigm as mentioned by Guedri et al, (2017) who used a 3hr/day for 14-day paradigm.

The unfolded protein response is the cellular response to misfolded proteins which results due to a stressful environment in which the cell resides. Such stressors can impact the quality of protein folding and thus the cell will attempt to repair, remove the insulting proteins, and finally, if the stressor is severe enough, commit itself to apoptosis. The first response in the UPR is the releasing of GRP78, which negatively regulates the UPR, from the sensor proteins (IRE1α, PERK, ATF6). GRP78, or BiP, is a chaperone that stabilizes misfolded proteins by selectively binding to exposed amino acids. In this sequence, ATF6 is one of the first responders as it is immediately released from the ER once BiP dissociates from it. ATF6 is then modified by a local Golgi apparatus to become spliced into its final form, an activated transcription factor. At this point, ATF6 migrates to the nucleus and begins transcribing more stabilizing chaperones as well as the transcript of *Xbp-1* (which is then spliced into an active transcription factor). Our study showed the enhanced expression of both of these genes which are responsible for the early and prolonged ER stress response. ATF6 has not been specifically linked to depression, as this line of research is fairly novel, however, its activity can be inferred in reports that show increased presence of chaperones in the temporal cortex of patients who died by suicide (Bown et al., 2000). The next portions of UPR activity are dependent upon the dimerization and oligomerization of PERK and IRE1α. The responses of these events (due to prolonged stress releasing more BiP from these sensors) include a net decrease in protein production, enhanced ERAD activity (removing of proteins from the ER) and activation of pro-inflammatory and apoptotic pathways (stress-severity dependent). ATF4, which is in a similar class of transcription factors as ATF6, further shuts down protein production and selectively transcribes CHOP. Unlike ATF6, an increased presence of ATF4 is not necessary to induce CHOP and its transcriptional activity. Once ATF4 is phosphorylated (through PERK and the eIF-p interaction), CHOP is selectively transcribed. ATF4s activity is shown by the manifestation of CHOP rather than by an explicit increase in its own presence as our results show (UPR physiology is extensively reviewed by Kaufman and Ron (Kaufman, 2002; Ron, 2002)). As evidence of the UPR activation, we not only showed a significant increase in expression of GRP94 in restraint rats, but we also evaluated the protein expressions of IRE1 and PERK in their total and phosphorylated forms. Both of these proteins showed upregulation in the restraint rats when compared to the controls. Further, the spliced variant of the *Xbp-1* gene (*sXbp-1*) was also analyzed and found to be significantly upregulated in the restraint rats.

Inflammation is a well-established factor involved in the pathophysiology of depression (Felger and Lotrich, 2013; Lotrich, 2012; Raison et al., 2006; Young et al., 2014). In this study, we expanded our findings of UPR in animal models of depression and further explored pro-inflammatory-primed environment facilitated by the UPR. We specifically examined if there was an interaction between the UPR and toll-like receptors. GRP94 is a chaperone protein that has been shown to be upregulated in response to cellular stress (Bown et al., 2000; Yang et al., 2007). GRP94 is involved in the proper folding and chaperoning of the toll-like receptors, namely TLR2, 4, 5, 7, and 9. These receptors are involved in the adaptive immune response and interact with and promote the production of pro-inflammatory cytokines. The toll-like receptors have been shown to be upregulated in patients with major depression; specifically TLRs 2, 3, 4, 5, 7 and 9, all of which are folded by GRP94 except TLR3 (Hung et al., 2016; Pandey et al., 2014). As with these studies, we

found that rats that underwent chronic restraint stress had significantly upregulated expression of TLRs 2, 4, 7, and 9 in the hippocampus. This coupled with the significant upregulation of GRP94 at the protein level helped establish the connection between increased UPR activity and TLR folding. Our study also suggests that there is a direct link between the UPR and the pro-inflammatory environment, which is well established in depressed patients and rodent models of depression (Felger and Lotrich, 2013; Goodall et al., 2010; Lotrich, 2012). To a certain extent, interplay with the UPR to inflammation has also been demonstrated (Kim et al., 2015). In the present study, we show that the correlation of increased expression in GRP94 and the aforementioned TLRs is tangibly observed in the Co-IP experiment. All studied TLRs showed a significant increase in interaction with GRP94 in the restraint groups when compared to the control group. This strongly suggests that the UPR and its downstream consequences are profoundly involved in the development of a pro-inflammatory environment in the hippocampus of depressed rats.

Each of the aforementioned TLRs has unique roles in mediating the inflammatory response and is intimately tied to inflammatory pathophysiology. TLR2 is a receptor that reacts to bacterial lipoproteins as well as lipoteichoic acid (Hung et al., 2014). Interestingly, this receptor has also been shown to be upregulated in several diseases including asthma and sepsis as well as psychiatric diseases like bipolar disorder and schizophrenia (McKernan et al., 2011). TLR2 works synchronously with the UPR along with TLR4 to induce IRE1α which increases the splicing of *Xbp-1*. This leads to the production of cytokines like interleukin-6 (IL-6), tumor necrosis factor (TNF), and interferon-β (IFN β). This works by TRAF6 interaction with IRE1 which ultimately attenuates IRE1 inactivation and dephosphorylation (Grootjans et al., 2016). To further demonstrate the connection between the UPR and inflammation activity, it has been shown that hyperactivation of IRE1α leads to activation, through phosphorylation of JUN-N terminal kinase (JNK) (Kaser et al., 2008), which is tied to many inflammatory responses like cytokines and growth factors as well as phosphorylation of transcription factors, critical in inflammation regulation (Roy et al., 2008).

TLR4, another receptor that is chaperoned by GRP94, is crucially important in working alongside TLR2 to act on the IRE1α pathway involved in the UPR. Interestingly, these TLRs also have a role in suppressing the ATF4-CHOP branch of UPR activity, thus providing a protective motif in returning to homeostasis after an initial insult to a cell (Woo et al., 2009). This is intriguing as our results show an increased expression of CHOP, and to a lesser extent, ATF4 (though not significantly). This suggests that the pathway is not inhibited and, despite TLR4 (and TLR2) upregulation, is very active in the hippocampus of restraint rats. Another interesting fact, however, is that a concentrated presence of endogenous heat shock proteins, like GRP78 and GRP94, can activate TLR4. This is an interesting tie to the UPR as it is responsible for increasing the presence of heat-shock proteins and chaperones (Kaufman, 2002; Takeda and Akira, 2005). Zacharowski and colleagues showed that the expression of TLR4 is necessary for the corticosterone-mediated stress response and further showed that this affects major depression and other psychiatric disorders like bipolar disorder (Zacharowski et al., 2006). It is, to date, one of the most well characterized members of the TLR family in terms of its role in disease and pathophysiology

(Hung et al., 2014; Pandey et al., 2014; Wu et al., 2015). Observing increased regulation of this protein should come as no surprise in our restraint rats.

An increased expression in TLR7 has been shown to be sufficient to induce depressive-like behavior in mice (Kubo et al., 2013). Like TLRs 2 and 4, TLR7 is also folded and chaperoned by GRP94. It has also been shown to be upregulated in depressed patients (Hung et al., 2014) while simultaneously, the expression of TLR7 is downregulated in response to antidepressants (Hung et al., 2016). In general, TLR7 is an intracellular receptor that reacts to nucleic acids in the cell. When activated, intracellular signaling pathways impinge upon pro-inflammatory signaling such as cytokines. These play an important role in cellular and innate immune response (Ramirez-Ortiz et al., 2015).

In normal conditions, TLR9 reacts to CpG DNA like that found in bacteria (Takeda and Akira, 2005). The result of TLR9 activation is the production and secretion of interferons like IFN-α and IFN-I (Hemmi et al., 2003; Lund et al., 2003). Per previous report (Hung et al., 2014), TLR9 has also been implications in major depression and is chaperoned by GRP94 of the UPR. Not only is TLR9 overexpression ameliorated by antidepressants (Hung et al., 2016), which shows how intimately tied it is to stress-induced disorders like depression, but it has also been found to have an active role in apoptosis, specifically in macrophages. Downstream of the activation of TLR9 also leads to sustained, elevated production of pro-inflammatory cytokines IL-1β, TNF-α, and IL-10 (Xiang et al., 2015). These cytokines are regulated by the UPR (Kim et al., 2015), and suggest cross-play between the upregulated UPR and increased expression of toll-like receptors in the context of the depressed brain.

5. CONCLUSION

Altogether, our findings suggest that not only UPR is active in the hippocampus of restraint rats but there is interplay between UPR activity and TLR expression, which may be critical in producing the pro-inflammatory environment that is signature of depression. Further studies will be needed to examine the functional response of these interactions by examining cytokines and inflammatory markers in hippocampus of these rats. In addition, it will be interesting to study the factors that contribute to the interaction of UPR and TLRs. While typical TLR signaling is ligand/receptor interaction, the precise nature of activation of many of the TLRs is not known as psychiatric disorders are not classic foreign-body invasions. Recent studies, however, have suggested that miRNAs, specifically let-7b, miR-21, and miR-29a, can act directly as ligands for TLRs like TLR7 and TLR8 (Chen et al., 2013). This is a potential direction for future research as increased TLR7 has already been shown to be sufficient to induce depressive-like behaviors in mice (Kubo et al., 2013).

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Highlights

- Expression of UPR associated genes were significantly increased in hippocampus of restraint rats, a model of stress-induced depression.
- mRNA and protein expression of inflammatory mediators TLRs 2, 4, 7, and 9 were significantly upregulated in hippocampus of restraint rats.
- There were significant elevations in the interactions between UPR chaperone protein GRP94 and TLRs in hippocampus of restraint rats.
- UPR and associated TLRs may be an integral part of increased inflammatory activity in depression.

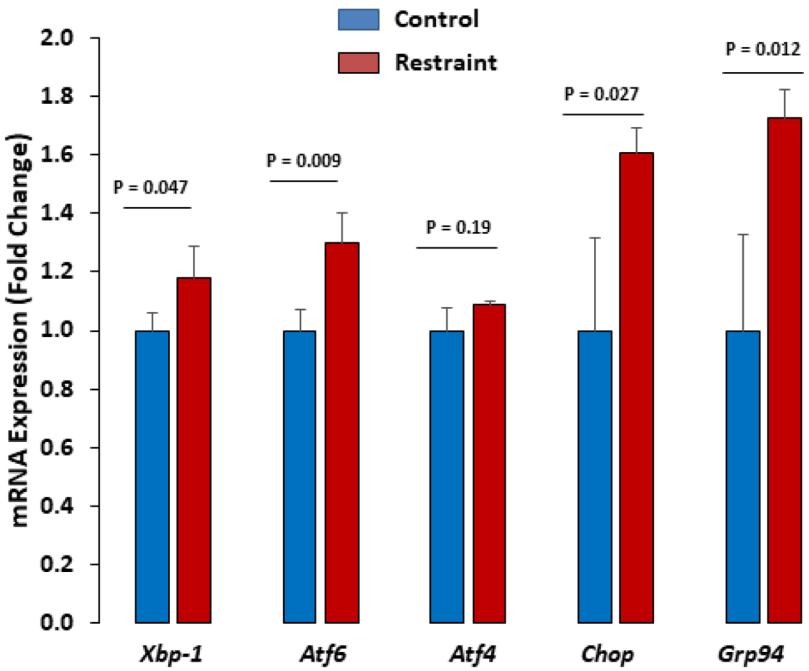

Figure 1.

mRNA expression of *Xbp-1*, *Atf6*, *Atf4*, *Chop*, and *Grp94* in hippocampus of control (n = 9) and restraint (n = 9) rats. Data are the mean ± SEM. The gene expression differences between groups is as follows: *Xbp-1* 17.8% increase; *Atf6* 29.9% increase; *Atf4* 8.6% increase; *Chop* 60.7% increase; and *Grp94* 72.7% increase. Significance was determined using a one-way student's t-test comparing control and restraint groups.

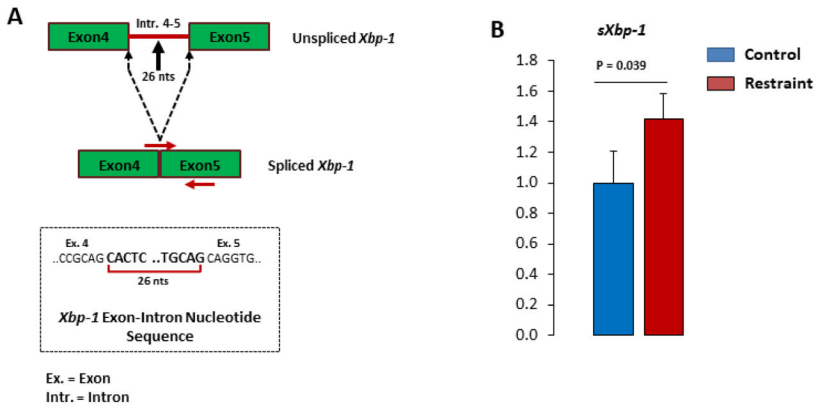

Figure 2.

mRNA expression of *sXBP-1* in hippocampus of control (n = 8) and restraint (n = 8) rats. **A.** schematic diagram showing the *sXbp-1* related fourth-fifth exons flanking the 26 nucleotides long intron 4–5 and splice junction spanning primer binding position. The lower panel shows the nucleotide sequences in relation to the unspliced *Xbp-1*. **B.** Bar diagram shows a 1.42 fold increase (p = .039) of *sXbp-1* expression in restraints compared to control group of rats. (→= *sXbp-1* forward primer, ←= *sXbp-1* reverse primer). Significance was determined using a one-way student's t-test comparing control and restraint groups.

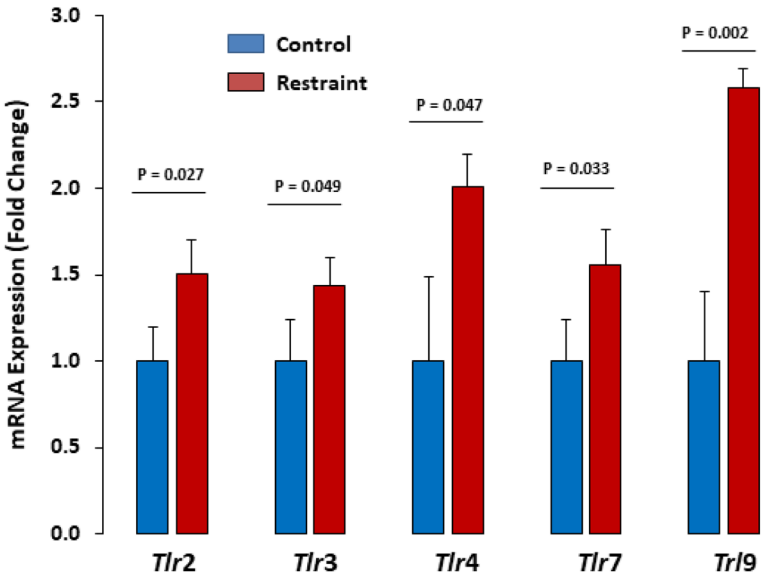

Figure 3.

mRNA expression of *Tlr2*, *Tlr3*, *Tlr4*, *Tlr7*, *Tlr9* in hippocampus of control (n = 9) and restraint (n = 9) rats, mRNA levels were determined by qRT-PCR. Data are the mean ± SEM. The gene expression differences between groups are as follows: *Tlr2* 50.1% increase; *Tlr3* 43.3% increase; *Tlr4* 100.0% increase; *Tlr8* 55.8% increase; and *Tlr9* 157.6% increase. Significance was determined using a one-way student's t-test comparing control and restraint groups.

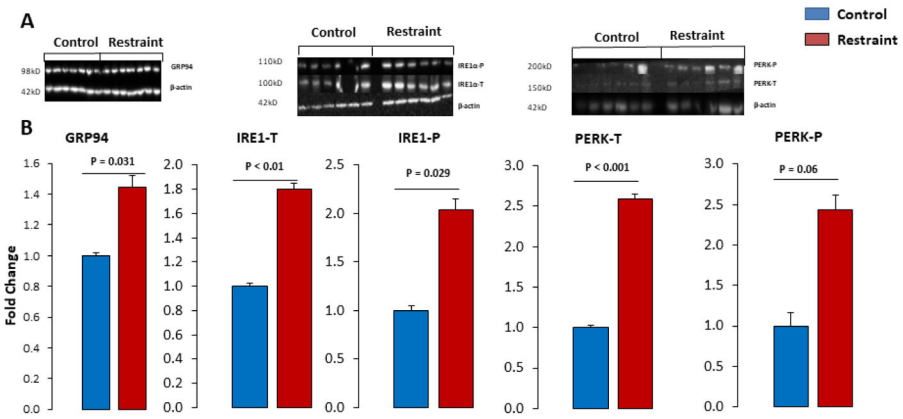

Figure 4.

Protein expression of different genes related to UPR pathway. **A.** Representative Western blots showing the effect of restraint stress on protein expression levels of GRP94, IRE1 and PERK in hippocampus. Changes in both total (T)) and phosphorylated (P) forms of IRE1 and PERK proteins are also included in this figure. **B.** Densitometric analysis of the three proteins (GRP94, IRE1 and PERK) including the total and phosphorylated forms of IRE1 and PERK were done by using ImageJ software. The representative bar diagrams indicate the changes observed between two groups (Control vs restraint) of rats when normalized with β-actin expression. For all five protein forms including GRP94 (n = 7/group) and two forms of IRE1 and PERK (n = 4/group), an overall increase was observed in restraint rats as compared to control group:GRP94 = 0.4 5-fold increase, $p = 0.031$; IRE1-T = 0.80-fold increase, $p < 0.01$; IRE1-P = 1.04-fold increase, $p = 0.029$; PERK-T = 1.59-fold increase, $p < 0.001$ and PERK-P = 1.44-fold increase, $p = 0.06$. All data are represented as ± S.E.M. (MW = Molecular Weight). Significance was determined using a one-way student's t-test comparing control and restraint groups.

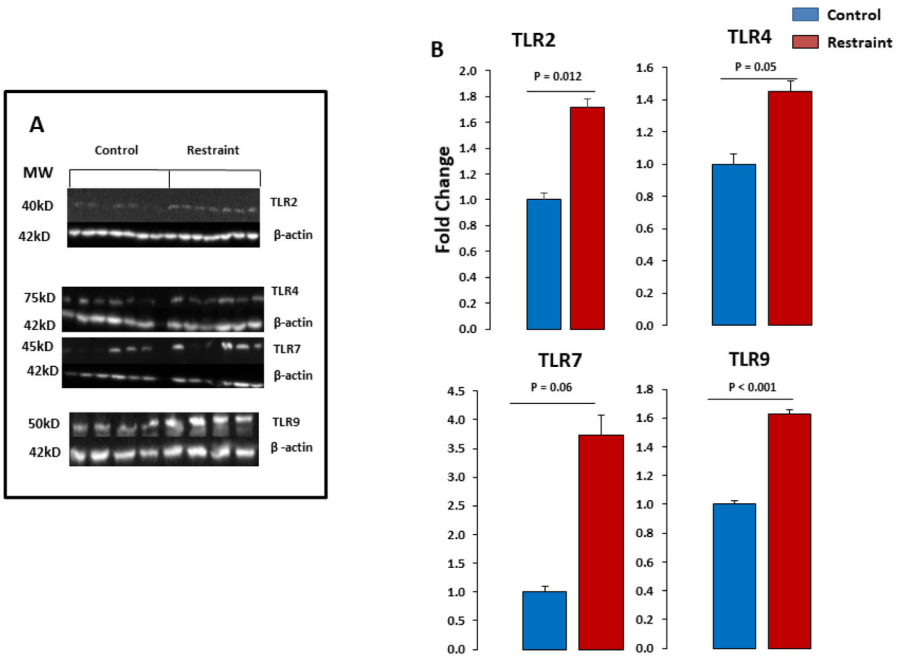

Figure 5.

Protein expression of different Tlr genes. **A.** Representative Western blots of TLRs 2, 4, 7, and 9 in control and restraint rats. **B.** Bar diagram showing fold change in protein expression of TLRs 2, 4, 7, and 9 in restraint rats compared with controls (n = 7/group). TLR2 showed a 0.71 fold increase ($p = 0.012$) in hippocampus of restraint rats compared to the control group. TLR4 showed a 0.5 fold increase ($p=0.05$) in the hippocampus of restraint rats compared to controls. TLR7 showed a 2.6 fold increase ($p=0.06$) in protein expression when compared to control rats. TLR9 showed a 0.63 fold increase ($p<0.001$) in expression when compared to control hippocampal tissue. Significance was determined using a one-way student's t-test comparing control and restraint groups.

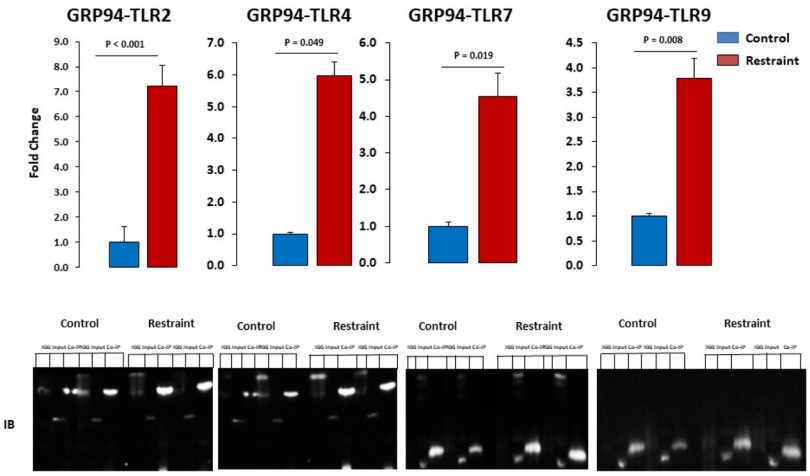

Figure 6.

Demonstration of in vivo protein-protein interaction between GRP94 and four different TLRs (2, 4, 7, and 9) as determined in hippocampus of control and restraint rats (n=4) following Co-IP assay. All the TLRs including 2, 4, 7 and 9 showed increased interaction with GRP94 protein in restraint rats as compared to control group. TLR2 and GRP94 showed a 6.21 fold increase ($p < 0.001$), TLR4 and GRP94 showed a 5.52 fold increase ($p = 0.049$), TLR7 and GRP94 showed a 4.54 fold increase ($p = 0.019$) and TLR9 showed a 2.79 fold increased interaction with GRP94 (0.008) in restraint rats compared to the control group. Significance was determined using a one-way student's t-test comparing controls to restraints.

Table 1

DNA oligo sequences used for qPCR based gene quantification

Genes	Primer sequences	
	Forward	**Reverse**
Grp94	5′-AAACGGCAACTCTTCGGTCA-3′	5′-TTAAGCTGAGGCGGAGCATC-3′
Atf6	5′-CGAGGGAGAGGTGTCTGTTTC-3′	5′-GTCTTCACCTGGTCCATGAGG-3′
Xbp-1	5′-CCACTTGGTACAGACCACTCC-3′	5′-AGACACTAATCAGCTGGGGG-3′
Atf4	5′-AAGGCAGATTCTCTCGCCAA-3′	5′-TTCTTCCCCCTTGCCTTACG-3′
Chop	5′-AGGAGAGAGAAACCGGTCCAA-3′	5′-GGACACTGTCTCAAAGGCGA-3′
Gapdh	5′-CACTGAGCATCTCCCTCACAA-3′	5′-TGGTATTCGAGAGAAGGGAGG-3′
Tlr2	5′-TGTTCCGGGCAAATGGATCA-3′	5′-GCCTGAAGTGGGAGAAGTCC-3′
Tlr3	5′-CGGTAACGATGCCTTCTCTTG-3′	5′-AGAGTGAGGGGTCAAACGCT-3′
Tlr4	5′-GAGGCAGCAGGTCGAATTGT-3′	5′-AGAAGATGTGCCTCCCCAGA-3′
Tlr7	5′-TGAGGGTATGCCACCGAATC-3′	5′-CCAATCTCGCAGGGACAGTT-3′
Tlr9	5′-CAGCCCTGACTAGGGACAAC-3′	5′-GTCGCTCGCTCTGAGCTATT-3′
sXbp-1	5′-GCTGAGTCCGCAGCAGGT-3′	5′-AGAGGCAACAGCGTCAGAAT-3′