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Innate immunity in the postmortem brain of depressed and suicide subjects: role of Toll-like receptors

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Abstract

Abnormalities of Toll-like receptors (TLRs) have been implicated in the pathophysiology of depression and suicide. Interactions of TLRs with pathogen-associated molecular patterns (PAMP) and damage-associated molecular patterns (DAMP) initiate signaling through myeloid differentiation primary response-88 (MyD88) and produce cytokines through the activation of the transcription factor nuclear factor kappa beta (NF-kB). We have earlier shown an increase in the protein and mRNA expression of TLR3 and TLR4 in the prefrontal cortex (PFC) of depressed suicide (DS) subjects compared with normal control (NC) subjects. To examine if other TLRs are altered in postmortem brain, we have now determined the protein and mRNA expression of other TLRs (TLR1, TLR2, TLR5, TLR6, TLR7, TLR8, TLR9 and TLR10) in the PFC of DS, depressed non-suicide (DNS), non-depressed suicide (NDS) and NC subjects. We determined the protein expression by Western blot and mRNA expression levels by real-time PCR (qPCR) in the PFC of 24 NC, 24 DS, 12 DNS and 11 NDS subjects. Combined with our previous study of TLR3 and TLR4, we found that the protein expression of TLR2, TLR3, TLR4, TLR6 and TLR10, and mRNA expression of TLR2 and TLR3 was significantly increased in the DS group compared with NC group. This study demonstrated that certain specific TLRs are altered in DS subjects, and hence those TLRs may be appropriate targets for the development of therapeutic agents for the treatment of suicidal behavior.

Keywords

Toll-like receptors (TLRs); cytokines; depression; suicide; postmortem brain; innate immunity

1. Introduction

Abnormalities of the immune function have been implicated in the pathophysiology of depression and suicide (Drexhage et al., 2011; Steiner et al., 2008). Cytokines, which are a heterogeneous group of messenger molecules produced by immunocompetent cells, play an

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Conflict of interest

All authors declare that they have no financial interests or potential conflicts of interest directly or indirectly related to this work.

important role in mediating immune functions. Evidence that cytokines might play an important role in inducing depression is derived from both animal and human studies, as described below

The administration of cytokines to animals and humans induces symptoms that have been termed as sickness behavior (Dantzer, 2001). This sickness behavior is characterized by symptoms such as weakness, malaise, restlessness, inability to concentrate, lethargy, decreased interest in surroundings and reduced food intake, all of which are depression-like symptoms (Dantzer, 2001).

About 30 to 40% of patients receiving chronic exogenous administration of IL-2 for renal cell cancer and metastatic melanoma experienced fatigue, anorexic sleep disorders, depressed mood, anxiety and cognitive dysfunction (Dantzer and Kelley, 2007). These symptoms are also seen in patients receiving chronic cytokine administration for hepatitis (Capuron et al., 2002). The depressive symptoms may be alleviated by the administration of antidepressants, such as paroxetine (Capuron et al., 2002). These symptoms appear to be prevented by prophylactic therapy (Dantzer, 2006). It has been shown that paroxetine has an effect on depressive symptoms but not on the fever, fatigue, and anorexia associated with sickness behavior (Dantzer, 2006).

In humans, the involvement of cytokines in the regulation of behavioral symptoms has been studied by the application of bacterial endotoxin LPS in human volunteers (Reichenberg et al., 2001). LPS, a potent activator of proinflammatory cytokines was found to induce fever, anorexia, anxiety, depressed mood and cognitive impairment, and the levels of anxiety, depressed mood, depression and cognitive impairment were found to be related to the levels of circulating cytokines (Miller et al., 2009; Reichenberg et al., 2002a; Reichenberg et al., 2002b; Reichenberg et al., 2001).

The other major line of evidence that cytokines may play a role in depression is derived from the observation that the levels of proinflammatory cytokines in the serum and cerebrospinal fluid (CSF) is increased in patients with depression (see review by (Dowlati et al., 2010; Hiles et al., 2012) and suicidal behavior (Black and Miller, 2015)

Depression and stress are major risk factors for suicidal behavior, and it has also been shown that stressful events cause changes in cytokine levels both in animals and humans (Kim and Maes, 2003; Minami et al., 1991). It is therefore not surprising that the role of cytokines has been studied, although to a limited extent, in patients with suicidal behavior. Janelidze et al. (2011) found that the levels of IL-6 and TNF-α were increased, and the levels of IL-2 were decreased in those patients who attempted suicide. Lindqvist et al. (2009) found increased levels of IL-6 in the CSF of suicide attempters compared to normal control subjects, and Nassberger and Traskman-Bendz (1993) found that patients with the history of suicide attempts have increased blood levels of soluble IL-2 receptors compared to healthy controls. Whereas there are several studies of cytokines in the serum of depressed and suicidal patients, there are only a few studies which suggest that abnormal immune function may be associated with the postmortem brain (Pandey et al., 2012; Shelton et al., 2010; Tonelli et al., 2008). Pandey et al. (2012) found increased expression of proinflammatory cytokines,

IL-1β, IL-6 and TNF-α, in the prefrontal cortex (PFC) of teenage suicide subjects. Tonelli et al. (2008) found increased expression of TH2 cytokines (IL-4 and IL-13) in the Brodmann area 11 of suicide subjects. Shelton et al. (2010), using the gene array method found some of the immune genes altered in the PFC of depressed subjects compared to controls. Abnormalities of the immune function have also been shown by studies of astrocytes and microgliosis in suicidal patients, including both the depressed and schizophrenic suicidal patient (Steiner et al., 2008).

Steiner et al. (2008) found increased microgliosis in the postmortem brain of suicidal subjects suffering with depression and schizophrenia, suggesting immune function abnormalities in suicide. Torres-Platas et al. (2011) found astrocyte hypertrophy in anterior cingulate white matter of depressed suicides [also, see review by (Mechawar and Savitz, 2016)]. These studies indicate abnormal immune function in depression and suicide postmortem brain. Torres-Platas et al. (2014a; Torres-Platas et al., 2014b) (2014b) found that the ratio of primed over ramified microglia was significantly increased in depressed suicides in the dorsal anterior cingulate cortex. However, there were no significant difference in overall microglial densities between suicides and controls.

The evidence for the inflammatory response in the CNS is derived from a PET study (Sandiego et al., 2015), who studied a neuroinflammatory response by administration of LPS, also called endotoxin in humans, with a PET radiotracer [^{11}C]PBR-28, which binds to the translocator protein, a molecular marker that is upregulated by microglial activation. LPS administration significantly increased [^{11}C]PBR-28 binding demonstrating microglial activation throughout the brain.

Recently, Holmes et al. (2018) used [^{11}C]R PK11195 PET and compared TSPO availability in different areas of the brain in MDD and control subjects and found significantly higher TSPO availability in anterior cingulate cortex (ACC) and a smaller increase in in PFC and insula of MDD subjects compared to controls suggesting of predominantly increased microglial activation in brains of depressed subjects,

The observed increase in cytokines in postmortem brain of suicide subjects could be related to increased transport of cytokines from the periphery into the CNS (Vitkovic et al., 2000) by crossing the blood-brain barrier (BBB) through several mechanisms such as (a) active transport through carriers (Dantzer et al., 2008); (b) passive diffusion through leaky regions of the BBB; (c) transmission along different vagal pathways (Banks, 2006; Capuron and Miller, 2011; Krishnadas and Cavanagh, 2012; Makhija and Karunakaran, 2013; Miller et al., 2009). This increase in postmortem brain level of cytokines may also be caused by increased synthesis of the cytokines in the brain (Szelenyi, 2001). The increased synthesis of cytokines in the brain may be related to abnormal innate immunity in the brain. Innate immunity in the brain is the initial microbial non-specific response that results in the rapid production of chemokines and inflammatory cytokines (Bsibsi et al., 2002; Rivest, 2009).

The key components of the innate immune system are the receptors known as pathogen recognition receptors (PRRs). The most important subgroup of PRRs is the family of Toll-like receptors (TLRs). These receptors have the ability to recognize pathogens or pathogen-

derived products, also known as pathogen-associated molecular patterns (PAMPs). They also recognize products from tissue damage, such as heat shock proteins (HSPs), known as damage-associated molecular pattern molecules (DAMPs). The interaction of these DAMPs or PAMPs with TLRs initiate signaling through myeloid differentiation primary response-88 (MyD88) causing the activation of nuclear factor kappa beta (NF-kB) and resulting in the production of cytokines and chemokines. Thus, increased cytokine levels in the PFC of suicidal subjects could be related to the abnormality of innate immunity because of the overexpression of TLRs.

TLRs are a family of innate immune system receptors that initiate signaling in response to PAMPs or DAMPs (Lehnardt et al., 2008; Pandey and Agrawal, 2006). The first TLR protein was discovered in drosophila melanogaster (Kawai and Akira, 2007; Valanne et al., 2011). Subsequently, many additional TLRs have been identified across diverse species, including the human brain (Kielian, 2006; Mishra et al., 2006; Takeda et al., 2003). Recent studies suggest that mammalian TLRs possess developmental roles during embryogenesis (Ma et al., 2006; Vijay-Kumar et al., 2010), as well as physiological and metabolic roles in adults (Okun et al., 2011). TLRs are expressed in a variety of mammalian immune system-related cell types, including B-cells (Iwamura and Nakayama, 2008), mast cells (Eriksson et al., 2006), macrophage, monocytes (Eriksson et al., 2006), dendritic cells, neutrophils and basophils (Sutmuller et al., 2007). They are also widely expressed in the CNS (Bsibsi et al., 2002; Kielian, 2006), and it has also been found that neurons (Lafon et al., 2006), microglia, and astrocytes (Bowman et al., 2003; Bsibsi et al., 2006; Tang et al., 2007) express TLRs. However, human neurons express only TLR-3 (Lafon et al., 2006).

It is therefore quite possible that, in addition to the transport of cytokines form the periphery to the CNS, increased proinflammatory cytokine levels in the suicide brain, as reported by us (Pandey et al., 2012), and by other investigators (Tonelli et al., 2008), may be related to increased levels and stimulation of some of the TLRs. Thus, one of the mechanisms by which the cytokines could be increased in the CNS may be related to the simulation of TLRs by agonists, such as LPS. Therefore, it is quite possible that increased levels of proinflammatory cytokines in the brain may be related to increased stimulation and activation of various TLRs in the brain through their interaction with PAMPs or DAMPs (Akira and Takeda, 2004). To test this hypothesis, we initially determined the protein and mRNA levels of two important TLRs, TLR3 and TLR4, in the PFC of depressed suicidal and depressed non-suicidal subjects (Pandey et al., 2014). We found that protein and mRNA expression levels of TLR3 and TLR4 were increased in the depressed suicide (DS) and depressed non-suicide (DNS) subjects. Since different TLRs recognize and respond to different PAMPs and DAMPs, it is possible that other TLRs may also be altered in the brain of depressed and suicidal subjects.

To test this possibility, we determined the protein and mRNA expression levels of other TLRs (i.e., TLR1, TLR2, TLR5, TLR6, TLR7, TLR8, TLR9 and TLR10) in the PFC of DS, DNS, non-depressed suicide (NDS) and normal control (NC) subjects. We studied two suicide groups (DS and NDS) and a non-suicide group (DNS) to also examine if TLRs abnormalities are primarily related to suicide or depression.

2. Materials and methods

2.1. Subjects and diagnoses

The study was performed in the PFC (Brodmann area 9 [BA9]) of 24 depressed suicide (DS) subjects, 12 depressed non-suicide (DNS) subjects, 11 non-depressed suicide subjects (NDS) and 24 non-psychiatric control subjects, hereafter referred to as normal control (NC) subjects. Brain tissues were obtained from the Maryland Brain Collection at the Maryland Psychiatric Research Center, Baltimore, Maryland. Tissues were collected only after a family member gave informed consent. All tissue from normal control and suicide subjects was grossly examined by experienced neuropathologists. Toxicology data were obtained by the analysis of urine and blood samples. All procedures were approved by the University of Maryland Institutional Review Board (IRB) and by the University of Illinois IRB.

2.1.1 Inclusion and Exclusion Criteria—The DS group consisted of subjects diagnosed with MDD. Subjects with bipolar illness were not included in this group. Also, subjects with a history of major medical disorders, neurological disorders (such as epilepsy) and HIV were also excluded.

2.2. Diagnostic method

Subject diagnosis was based on the Structured Clinical Interview for DSM-IV (SCID I) (First et al., 1997). At least one family member and/or a friend, after giving written informed consent, underwent an interview. Diagnoses were made by a consensus of two psychiatrists from the data obtained in this interview, medical records from the case, and records obtained from the Medical Examiner's office. Normal control subjects were verified as free from mental illnesses using these consensus diagnostic procedures.

2.3. Determination of mRNA levels

2.3.1. RNA extraction—Total RNA was extracted from 100 mg of tissue using TRIZOL (Invitrogen, Thermo Fisher Scientific Inc., Waltham, MA) reagent, which was followed with DNase treatment according to the manufacturer's instructions. The RNA concentration and purity was determined by measuring the optical density with NanoDrop®ND-1000 (NanoDrop Technologies, LLC, Wilmington, DE) and using the 260/280 nm ratio with expected values between 1.8 and 2.0. The RNA quality was assessed using an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) to determine the RNA integrity number (RIN).

2.3.2. mRNA quantitation by real-time PCR—Real-time PCR was performed using Pre-designed Taqman gene expression assays (Applied Biosystems, Foster City, CA) for all target and housekeeping genes, on MX3005p sequence detection system (Agilent). The TaqMan assay IDs are in Table 1. To determine the stability and optimal number of housekeeping genes we used geNORM version 3.4 (PrimerDesign Ltd, UK) according to the manufacturer's instructions (Vandesompele et al., 2002) and tested twelve commonly used reference genes of different functional classes in 10 samples from each test group. The average gene-stability measure (M), ranked β-actin and GAPDH as the most stable genes in our samples. PCR efficiency was tested over 5-log dilution series and confirmed that β-actin,

GAPDH and all target genes had similar amplification efficiencies. For each primer/probe set, the PCR reaction is carried out using 10 μl of cDNA diluted 1:10 fold. Each qPCR plate includes a “no reverse transcriptase” and “no template” control to eliminate non-specific amplification, one sample is run on a gel to confirm specificity and samples were run in triplicates. Target gene qPCR data is normalized to the geometric mean of β-actin and GAPDH and is expressed relative to the control samples using $2^{-(\Delta\Delta Ct)}$ method.

2.4. Determination of protein expression of TLRs by Western blot

Since TLRs are primarily present in the membrane fraction, the protein expression of TLRs was determined by the Western blot method in membrane fractions, as described by us earlier (Dwivedi and Pandey, 2000).

Quantitation of TLR1, TLR2, TLR5, TLR6, TLR7, TLR8, TLR9, and TLR10 in membrane fraction by Western blot—Immunolabeling of TLRs was determined as described in a previous study (Dwivedi and Pandey, 2000). Briefly, equal volumes of membrane fractions isolated by this procedure (30 μg of protein in 20 μL) were loaded onto 7.5% (weight per volume) polyacrylamide gel and electrophoresed. The blots were initially developed using monoclonal anti-TLR2 (1:1000 dilution) and polyclonal anti-TLR1, TLR5, TLR6, TLR7, TLR8, TLR9, and TLR10 antibody (1:1000 dilution) and subsequently using β-actin monoclonal antibody (1:5000 dilution). The levels of TLR1, TLR2, TLR5, TLR6, TLR7, TLR8, TLR9, and TLR10 proteins were calculated as a ratio of the optical density of the TLR antibody of interest to the optical density of β-actin antibody. The antibodies for TLR1, TLR2, TLR5, TLR6, TLR7, TLR8, TLR9, and TLR10 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, California, USA), and had molecular weights of 90 kDa, 100 kDa, 110 kDa, 96 kDa, 121 kDa, 120 kDa, 113 kDa and 90 kDa, respectively.

2.5. Statistical analysis and effect of confounding variables

We analyzed the data using SAS 9.4 statistical software package. We used ANCOVA (Proc GLM) to jointly compare NC, DS, DNS, and NDS subjects adjusting the effects of age, gender, postmortem interval (PMI), brain pH, presence of antidepressants (yes or no), non-psychotropic medication (yes or no) and the use of ethanol (yes or no). For post-hoc multiple comparisons, we used Bonferroni t Tests to adjust the type I error rates, and we reported mean differences (mean-diff) and confidence interval (CI) to test the significance at the 0.05 level.

We conducted a separate regression analysis for suicide group only, as the covariate suicide method is relevant only for the two suicide groups (i.e., DS and NDS). In this analysis, we included all the covariates mentioned above along with the suicide method (violent or non-violent) to find their effectiveness for various protein and mRNA expressions. In addition we tested whether depression and suicide have independent (and possibly interactive) effects on the dependent variables by running a simple regression model by creating two dummy variables – “depression” and “suicide” from three groups – DS, DNS and NDS.

3. Results

3.1. Characteristics of study subjects

The demographic and the clinical characteristics of individual DS, DNS, NDS and NC subjects are given in Table 2. In addition, a summary of different variables such as age, gender, race, PMI, brain pH, suicide method, and the presence of antidepressants or ethanol at the time of death for each of these four groups is shown in Table 3.

Several of the DS subjects also had some co-morbid diagnoses that included mostly ethanol and polysubstance abuse, and in one case, panic disorder. DNS subjects had axis II diagnoses that included polysubstance and ethanol abuse, while NDS subjects had different psychiatric disorders that included adjustment disorders, substance abuse, and in one case a schizoaffective disorder.

In this study we determined the protein and mRNA expression of TLR1, TLR2, TLR5, TLR6, TLR7, TLR8, TLR9 and TLR10 in the PFC of DS, DNS, NDS and NC subjects and have combined these results with the protein and mRNA expression of TLR3 and TLR4 determined in the same cohort of DS, DNS, NDS and NC subjects in our previous study (Pandey et al., 2014).

3.2 Effect of covariates on TLRs expression in the PFC of DS, DNS, NDS and NC subjects

We first examined the effect of covariates on the TLRs expression using generalized linear model (Proc GLM in SAS) for each outcome measure. We excluded from this analysis the suicide method as a covariate, because it is relevant only for the two suicide groups (i.e., DS and NDS). ANCOVA analysis showed no significant effect of age on mRNA expression of any of the TLRs, except that age has a significant effect on protein expression of TLR7 ($t = 2.33$, $p = 0.02$). There was a significant effect of PMI on the TLR7 expression of mRNA ($t = 2.20$, $t = 0.03$) and protein ($t = -3.56$, $p = 0.0007$). Brain pH has significant effect on protein expression of TLR2 ($t = 3.02$, $p = 0.004$), and TLR10 ($t = 2.61$, $p= 0.01$) but no effect on mRNA expression of any of the TLRs. Gender has a significant effect only on protein expression of TLR10 ($t = 2.11$, $p = 0.04$).

Ethanol abuse had a significant effect on protein expression of TLR7 ($t = -2.82$, $p = 0.006$) and on mRNA expression of TLR9 ($t = 2.03$, $p = 0.04$). Non-psychotropic medication has a significant effect on protein expression of TLR10 ($t = -2.02$, $p = 0.04$). Antidepressants had no effect on either protein or mRNA expressions of any of the TLRs studied.

3.3. Protein expression levels of TLRs in the PFC of DS, DNS, NDS and NC subjects

The protein expression of TLRs was determined using Western Blot. A representative immunoblots showing the protein expression of TLRs in two NC, two DS, two DNS and two NDS subjects are shown in Figure 1. As the immunoblots suggest, there appears to be increased expression of TLR2, TLR6, TLR7 and TLR10 in DS subjects. There did not appear to be increases in TLR1, TLR5, TLR8, or TLR9 in DS subjects. The protein expression of all the TLRs is shown in Figure 2.

We compared the expressions of TLRs between the groups by performing ANCOVA. The ANCOVA F-tests showed that the above-mentioned four groups differ significantly in protein expression of TLR2 [F (10,63) = 4.57, p < 0.0001], TLR3 [F (10,64) = 3.73, p < 0.0006], TLR4 [F (10,63) = 2.03, p = 0.04], and TLR6 [F (10,60) = 3.29, p = 0.0017], TLR7 [F (10,60) = 3.47, p = 0.0012], TLR10 [F (10,60) = 4.11, p = 0.0002] controlling for the other predictors. However protein expression of TLR1 [F (10,63) = 1.03, p = 0.43], TLR5 [F (10,63) = 0.75, p = 0.67], TLR8 [F (10,60) = 1.61, p = 0.12] and TLR9 [F (10,60) = 1.17, p = 0.33] did not differ significantly among the four groups.

For multiple group comparisons, we used the Bonferroni (Dunn) t Tests. The results showed significant differences between DS and NC {TLR2 [mean-diff = 0.37, CI = (0.13, 0.61)], TLR3 [mean-diff = 0.35, CI = (0.12, 0.58)], TLR4 [mean-diff = 0.38, CI = (0.04, 0.71)], TLR6 [mean-diff = 0.56, CI = (0.20, 0.91)] and TLR10 [mean-diff = 0.36, CI = (0.01, 0.71)]}, NDS and NC {TLR3 [mean-diff = 0.46, CI = (0.16, 0.75)], TLR6 [mean-diff = 0.59, CI = (0.13, 1.04)] and TLR7 [mean-diff = 0.31, CI =(0.0, 0.62)]}, DS and DNS {TLR2 [mean-diff = 0.42, CI = (0.12, 0.72)], TLR3 [mean-diff = 0.34, CI = (0.05, 0.63)], and TLR6 [mean-diff = 0.58, CI = (0.13, 1.03)]}, NDS and DNS {TLR3 [mean-diff = 0.45, CI = (0.11, 0.80)], TLR6 [mean-diff = 0.61, CI = (0.08, 1.14)]},

3.4. Gene expression of TLRs in the PFC of DS, DNS, NDS and NC subjects

In order to examine if there are changes in the mRNA expression of TLRs we also determined the mRNA expression of these TLRs in DS, DNS, NDS and NC subjects (Fig. 3). The overall group effect using ANCOVA model for the GLR showed that the mRNA expression of TLR2 [F (10,61) = 2.23, p = 0.03], TLR3 [F (10,58) = 1.93, p = 0.05], TLR4 [F (10,55) = 1.97, p = 0.05], TLR7 [F (10,61) = 4.79, p < 0.0001] differed significantly between the groups. However, these three groups did not differ significantly in the mRNA expression of TLR1 [F (10,61) = 0.90, p = 0.54], TLR5 [F (10,61) = 0.92, p = 0.53], TLR6 [F (10,61) = 1.11, p = 0.37], TLR8 [F (10,61) = 0.98, p = 0.47] and TLR9 [F (10,61) = 1.03, p = 0.43],

Multiple group comparisons (Bonferroni t Test) revealed that there are significant differences between NC and DS {TLR2 [mean-diff = 0.38, CI = (0.06, 0.71)], TLR3 [mean-diff = 0.32, CI = (0.02, 0.62)]}, NC and NDS {TLR7 [mean-diff = 0.87, CI = (0.47, 1.27)]}, NC and DNS {TLR3 [mean-diff = 0.39, CI = (0.00, 0.79)], TLR4 [mean-diff = 0.50, CI = (0.03, 0.96)] and TLR7 [mean-diff = 0.43, CI = (0.01, 0.85)]}.

3.5. Correlation in the protein and mRNA expression of various TLRs

In order to examine the co-expression of various TLRs in the PFC, we determined the Pearson correlation coefficient in the protein and mRNA expression of various TLRs in the PFC of NC subjects. The observed correlations are described for protein in the supplementary Table S1 and for mRNA in the Table S2. We found that there was a significant correlation between protein expression of TLR1, TLR3 and TLR4; between TLR2 and TLR5; between TLR3, TLR1 and TLR4; between TLR4, TLR1, TLR3, and TLR7; between TLR5 and TLR2; and between TLR7 and TLR4. In contrast, there were no

significant correlations in protein expression of TLR8, TLR9 and TLR10 with any other TLRs (see supplementary Table S1)

When we examined the correlation of mRNA expression between various TLRs, we found that there was a significant correlation of mRNA expression between TLR1, TLR5, TLR6 and TLR7; between TLR2 and TLR6; between TLR3 and TLR4; between TLR4 and TLR3; between TLR5, TLR1 and TLR6; between TLR6, TLR1, TLR5, TLR9 and TLR10; between TLR7 and TLR1; between TLR9, TLR5, TLR6 and TLR10; and between TLR10, TLR6 and TLR9 (see supplementary Table S2).

These results suggest that in terms of protein expression, the TLR1, TLR3, TLR4, and probably TLR7 are highly expressed together and for mRNA, TLR1, TLR5, TLR6 and TLR7 and TLR6, TLR1, TLR5, TLR9 and TLR10 are highly co-expressed together. Whether the co-expression of these TLRs either in the protein or mRNA expression has any significance in their dysregulation in suicide is unclear at this time.

Based on the results of correlation between protein and mRNA expression of TLRs, we performed an additional analysis to check the dependency of mRNA using mRNA data as a covariate along with the other variables. None of TLRs in mRNA expression are significant while predicting corresponding protein TLRs. However we observed that the significant differences exist between DS and NC in TLR2, TLR3, TLR4, TLR6 and TLR10, between NC and NDS in TLR3. These results support our analysis in section 3.3.

3.6. Mean RIN Values and relative abundance and expression of TLRs in the brain

The mean RIN (RNA integrity number) values for each group are summarized in Table 3. There were no significant differences in mean RIN values between the four groups.

Since the levels of some TLRs were increased in the DS group, but not in the DNS group, we examined if this was due to the lower expression levels of some of the TLRs in the brain. The relative abundance of the mRNA levels of TLRs in NC subjects is shown in Figure 4. The lowest mRNA expression of TLRs was found in TLR3, TLR6 and TLR8, and the highest in TLR1 and TLR4. However, TLR3 and TLR4 mRNA levels were abnormal in the DS and DNS groups. Therefore, it appears that differences in TLRs found in DS and other groups are probably not related to relatively lower abundance of a particular TLR.

3.7. Effect of suicide method, antidepressants, non-psychotropic medications and ethanol on TLRs in two suicide groups

Since we observed increased protein and mRNA expression of some TLRs, like TLR2, TLR3, TLR4, TLR6 and TLR7, we examined if this increase in TLRs in suicide group is related to previous antidepressant treatment, suicide method, non-psychotropic medication treatment or ethanol use. We tested the effect of ethanol, non-psychotropic medication and suicide method for the two suicide groups (i.e., DS and NDS) and the effect of antidepressants for the DS group only. In the DS group, 11 out of 24 subjects were on antidepressant treatment at the time of death, and 13 were free of antidepressant medication (see Table 3). When we compared protein and mRNA expression of TLRs between antidepressant-treated and antidepressant-free DS subjects, we found that there was a

significant difference in the protein but not the mRNA expression of TLR8 (t = −2.21, p = 0.04). For the suicide groups (DS and NDS), there are significant differences between the effect of the use vs. no use of nonpsychotropic medication on the mRNA expression of TLR1 (t = −2.36, p = 0.02), TLR5 (t= −2.54, p = 0.02), and TLR 8 (t = 2.09, p = 0.04). The suicide method (i.e. violent vs non-violent) and ethanol use have a significant effect on the mRNA expression of TLR8 (t = 2.16, p = 0.04) and TLR1 (t = 2.12, p = 0.04), respectively.

3.8 Effect of depression and suicide on TLRs

To study the effect of depression and suicide on TLRs, we ran a simple regression model by creating two dummy variables depression and suicide from three groups – DS, DNS and NDS. Our analysis showed that suicide has an independent effect on protein expression of TLR3 (t = −3.07, p = 0.0036), TLR4 (t = −2.35, p = 0.0233), TLR6 (t = −2.09, p = 0.04). No independent effect of suicide or any interaction between suicide and depression have been found on protein expression of other TLRs. Depression has an effect only on mRNA expression of TLR7 (t = −3.51, p = 0.001).

4. Discussion

In this study we determined the protein and mRNA expression of TLR1, TLR2, TLR5, TLR6, TLR7, TLR8, TLR9 and TLR10 in the PFC of DS, DNS, NDS and NC subjects. In a previous study, we have determined protein and mRNA expression of TLR3 and TLR4 in the same cohort of DS, DNS, NDS and NC subjects (Pandey et al., 2014). Combining the results from these two studies, the main finding was that the protein expression of TLR2, TLR3, TLR4, TLR6 and TLR10 was significantly increased in the DS group compared with the NC group using the Bonferroni (Dunn) t Test. The protein expression of TLR3, TLR6 and TLR7 was significantly increased in the NDS group compared with NC group, while none of the TLRs had increased protein levels in the DNS group compared with NC group.

The mRNA expression of TLR2 and TLR3 was significantly increased in the DS group compared with the NC group, TLR3, TLR4 and TLR7 were significantly increased in the DNS group compared with NC group, and only TLR7 was increased in the NDS group compared with the NC group.

These observations suggest upregulation of specific TLRs in the DS and NDS groups and to a certain extent in the DNS group, thus suggesting abnormal innate immunity in suicide. Since abnormalities of some of the TLRs were also observed in the DNS group, it is not clear from this study if the dysregulation of innate immunity is specific to suicide or related to diagnosis, such as depression.

In these studies, we also found that whereas both protein and mRNA expression was increased in the DS and NDS groups there was some dissociation between abnormal protein and mRNA expression of some TLRs. For example, in the DS group protein expression of TLR2, TLR3, TLR4, TLR6 and TLR10, but mRNA expression of only TLR2 and TLR3 was increased compared with the NC group. Similarly, in the NDS group the protein expression of TLR3, TLR6 and TLR7 but mRNA expression of only TLR7 was significantly increased compared with the NC group.

Although the reasons for dissociation between TLR3, TLR4 and TLR7 mRNA and protein expression in DNS subjects are unclear, a similar dissociation was reported by Bosisio et al. (2002) for TLR4 receptors activated by lipopolysaccharide (LPS). They found that LPS has divergent effects on TLR4 expression at the mRNA and protein levels with upregulation of mRNA and downregulation of protein. Our observation of increased mRNA but not protein in DNS is similar to that observation. There may also be other reasons why changes in mRNA levels do not match changes in protein levels. For example, steady state mRNA levels are regulated by the balance between rate of synthesis and rate of degradation. The same is also true of protein levels. The changes in mRNA levels for a particular gene reflect changes in transcription of mRNA and its half-life. Additional processes, including RNA splicing, polyadenylation, length of polyA tail, miRMA expression and the expression of other noncoding RNAs, all influence the mRNA half-life. Some of the long non-coding RNAs being discovered can act as endogenous antisense transcripts. Collectively these factors impact RNA stability. Any changes in mRNA or protein levels reflect the balance of these factors acting to affect mRNA half-life.

Among the covariates, besides PMI, brain pH and age, the two other important covariates were the use of antidepressants and ethanol, especially in the DNS group. However, using antidepressants and alcohol as covariates, no significant effect of antidepressants and ethanol was observed.

However, our failure to observe differences in some of the TLRs in the DNS group, as we observed in the DS group may, in part, be related either to depression or suicide. However, it is also possible that one of the factors that may cause differences in TLR expression between DS and DNS groups may be related to previous chronic exposure to antidepressants. Antidepressant treatment has been shown to decrease or normalize the levels of several TLRs in the PBMC of depressed patients, as reported by Hung et al. (2016), where they found that antidepressant treatments completely normalized TLR3, TLR5, TLR7, TLR8 and TLR9 levels, whereas TLR1, TLR2, TLR4, and TLR6 were decreased below normal levels. However, although some subjects in the DS group were also treated with antidepressants, we did not find any significant effect of antidepressant treatment in this group. This may be related to the small number (n = 11) of DS subjects who were treated with antidepressants resulting in insufficient statistical power. In the DNS group, almost all the subjects were treated with antidepressants, most probably chronically. So, it is not clear if the differences between DS and DNS are related to suicide or to the antidepressant treatment. Consequently, the role of diagnosis in abnormal expression of TLRs cannot be completely ruled out.

To further examine the effect of depression and suicide on TLRs, we ran a simple regression model as described in the Results (section 3.8). The results showed a significant effect of suicide on protein expression of TLR3, TLR4 and TLR6. Depression has an effect on mRNA expression of TLR7. These results suggest that TLRs interact more with suicide than with depression.

There is a strong relationship between the CNS and the immune system, perhaps mediated by the innate immune system (Akira and Takeda, 2004; Rivest, 2009). There are two types of immune systems. One is known as the innate immune system and the other one as

adaptive immune system. The adaptive immune system is able to recognize and remember specific pathogens and then mount an attack each time that pathogen is encountered (Akira et al., 2006). The innate immune system responds to specific signals, which are derived from pathogens or other danger signals, such as bacterial wall components like LPS and the components from tissue damage such as ATP, uric acid and HSPs (Kawai and Akira, 2007). TLRs bind some of these components and then initiate a signal transduction ultimately resulting in the production and the release of the proinflammatory cytokines and chemokines in order to neutralize pathogens and initiate tissue repair (Kumar et al., 2011).

TLRs are the major family of PRRs in the cells (Rock et al., 1998) and they are key first molecules required for the induction of innate immune responses to various microbial ligands and endogenous host factors released by damaged cells (Janeway and Medzhitov, 2002). TLRs are type-1 transmembrane proteins that consist of 11 functional members and are classified into two subgroups based on their subcellular localization. One group consists of TLR3, TLR7, TLR8, TLR9 and TLR10 and they are expressed in the intracellular compartment such as the endoplasmic reticulum, endosomes, lysosomes, and endolysosomes (Takeda and Akira, 2005). On the other hand, TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11 are generally found on the surfaces (Akira et al., 2006). Intracellular TLRs recognize nucleic acid-based PAMPs and are particularly specialized in viral recognition, whereas cell surface TLRs sense mainly microbial membrane components, such as glycolipids, lipopeptides and fragilins of various organisms, including bacteria (Kawai and Akira, 2007). Different TLRs recognize specific PAMPs and DAMPs [for review see Takeda and Akira, (2005)]. Among the TLRs, the TLR4 mainly recognizes LPS form bacteria (Akira et al., 2006; Poltorak et al., 1998; Qureshi et al., 1999). TLR4 also recognizes a variety of other ligands from viruses, fungus and mycoplasma (Piccinini and Midwood, 2010). Many of the endogenous substances, such as high mobility group protein-1 (HMGB-1), heparin sulfate, are some of the typical DAMPS that act as TLR4 agonists (Piccinini and Midwood, 2010). The TLR2 responds to a wide array of molecules with triglyceride moieties proteins, polysaccharides, and the major TLR2 ligands include lipoproteins, highly expressed in the outer membrane of gram-positive bacteria (Oliveira et al., 2014; Takeuchi et al., 1999). TLR3 senses and recognizes double-stranded RNA (dsRNA), an intermediate generated during most viral infections, and acts as a sentinel agonist against viruses (Alexopoulou et al., 2001). The activation of TLR3 and TLR4 by their agonists results in the secretion of the microbial peptides and proinflammatory cytokines, such as TNF-α and IL-6 which contribute to an inflammatory response to clear viruses and bacteria, respectively in the CNS function (Alexopoulou et al., 2001). TLR-7 and TLR-8 require RNA from single stranded viruses (Diebold et al., 2004; Heil et al., 2004). The summary of TLRs and their ligands is shown in Table 4.

The specificity of agonist recognition by TLRs also depends on TLR dimerization. For example, most TLRs form homodimers; however, only TLR2 forms heterodimers with TLR1 and TLR6, as well as with CD36 (cluster of differentiation 36). TLRs may also be divided into groups based on PAMPs they recognize. TLR1, TLR2, TLR4 and TLR6 recognize lipids, TLR4 recognizes LPS, as mentioned before. TLR2 dimerizes with TLR1 to recognize triacylated lipopeptides, and with TLR6, it recognizes several PAMPs (Akira et al., 2006).

TLRs are expressed in many tissues and organs, but they are also highly expressed in several cells of the CNS, which include microglia (TLR1 to TLR9), astrocytes (TLR1 to TLR5 and TLR9) and neurons (TLR3). Among these TLRs, TLR3 is the only TLR which is expressed in the neuron (Lafon et al., 2006). TLRs also play an important role in neurodevelopment and neurodegeneration, and are expressed in the developing as well as in the adult brain and play a role in plasticity. They also play an important role in neuronal apoptosis thought the inhibition of neurite outgrowth (Ma et al., 2006).

TLRs mediate many beneficial effects, which include developmental and adult neuroplasticity (Okun et al., 2011). TLR2 and TLR4 are expressed in adult NPC and have been shown to regulate hippocampal neurogenesis in the adult brain (Rolls et al., 2007).TLR2, TLR3 and TLR4 are expressed in hippocampal neurons (Okun et al., 2010; Rivest, 2009), suggesting that this could modulate cognitive attributes. These TLRs can modulate cognitive functions and it has been shown that TLR3 deficient mice exhibited enhanced hippocampus-dependent working memory, whereas developmental TLR4 deficiency enhanced spatial reference memory acquisition and memory retention and impaired contextual fear learning (Okun et al., 2011). TLR activation, either due to infection or stress, may cause prolonged inflammation in the brain, which could subsequently lead to progressive neurotoxicity and neurodegeneration (Lehnardt et al., 2008). TLRs can act alone or heterodimerize to create specific responses to a given stimulus (Hayward and Lee, 2014).

Since TLRs are the main mediators of the innate immunity in the CNS, it is crucial to examine if the expression of TLRs is altered in psychiatric patients. Although ours is the first study of TLRs in the postmortem brain of psychiatric patients (Pandey et al., 2014), TLRs have been studied in the blood of psychiatric patients. For example, Garcia-Bueno et al. (2016) studied the protein expression of TLR4, MyD88, and NF-kB in the blood and PFC of patients with schizophrenia. They found an increase in the protein expression of TLR4 in the PFC of schizophrenic patients compared with normal control subjects. The expression of MyD88 was also increased in the PFC of schizophrenic patients (Garcia-Bueno et al., 2016; Garcia Bueno et al., 2016).

The levels of the TLRs have also been studied in the blood of depressed patients by Hung et al. (2014). They found that the mRNA levels of TLR3, TLR4, TLR5 and TLR7 were significantly increased in the peripheral blood of depressed patients, but had lower expression of TLR1and TLR6 in patients with major depression. Our results of TLRs in the brain are also similar as well as dissimilar to those observed by Hung et al. (2014). For example, we found that the mRNA expression of TLR2, TLR3, TLR4, TLR6, and TLR7 was increased in the PFC of depressed subjects. On the other hand, in the peripheral blood there were increases in TLR3, TLR4, TLR5, and TLR7, and lower expression of TLR1 and TLR6.These differences could be related to the tissue used -- peripheral blood vs. postmortem brain. Although it is important to study the TLRs in the peripheral blood for their possible use as biomarkers, the determination of the TLRs in the brain may be quite relevant to study the innate immune system in the CNS.

5. Conclusion

Among the TLRs we studied in the PFC, our observation that the protein and mRNA expression of TLR2 and TLR3 was increased in DS subjects is intriguing. As discussed earlier, TLR2 is very important and plays a role in cognitive function and brain function, responds to certain pathogens, and is abnormal in Alzheimer's disease. Its abnormality in DS brain may be crucial and play a role in the pathogenesis of suicide and depression resulting in increased formation of cytokines.

6. Limitations

The main limitation of this study is that we do not have information on the body mass index (BMI) and that most of the DNS subjects were on antidepressant treatment at the time of death. It is therefore quite possible that the difference between DS and DNS may be related to antidepressant treatment. However, in the DS group we did not observe differences in the expression of TLRs between those subjects who were on antidepressants compared with those subjects who were antidepressant-free, as described in the results section.

Another limitation is the lack of data on the history of suicides in the DNS group. In the DNS group, the depressed subjects died a natural death. It is quite possible that some of the subjects may have had a history of previous suicide attempts or suicide ideation; however, we do not have adequate information on this to examine if previous suicide attempts have any significant effect on TLRs expression. However, in this study we examined the effect of completed suicide, which may be different than suicide attempts or suicidal ideation. Nonetheless, this is one of the limitations of the study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- The expression of several TLRs is increased in prefrontal cortex of depressed suicide subjects
- There is a dissociation in gene and protein expression of several TLRs in depressed suicide subjects
- These findings suggest an abnormality of innate immunity in depression and suicide

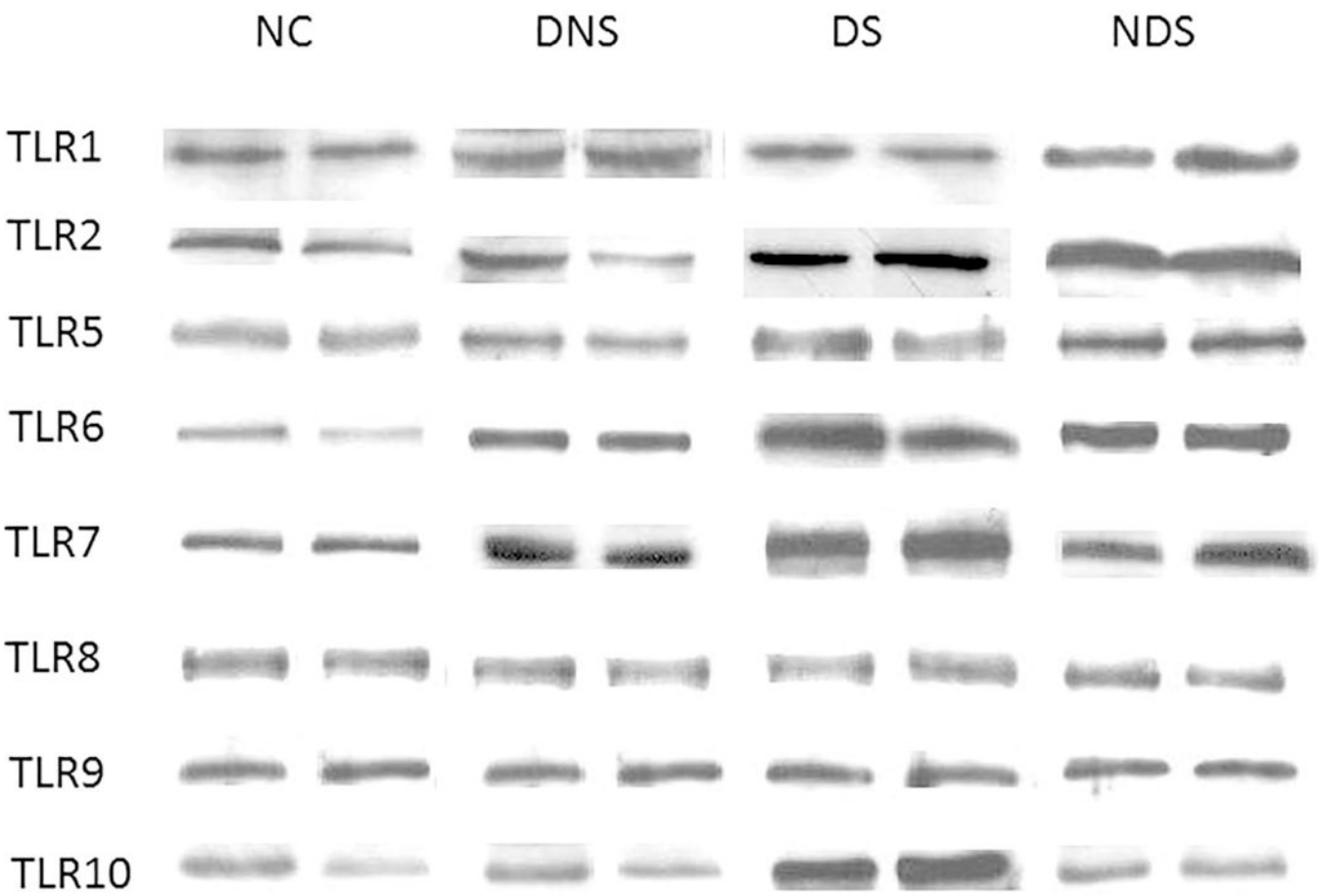

Figure 1.

Representative Western blots showing the immunolabeling of TLR1, TLR2, TLR5, TLR6, TLR7, TLR8, TLR9 and TLR10 in the prefrontal cortex (PFC) of two normal control (NC), two depressed suicide (DS), two depressed non-suicide (DNS) and two non-depressed suicide (NDS) subjects.

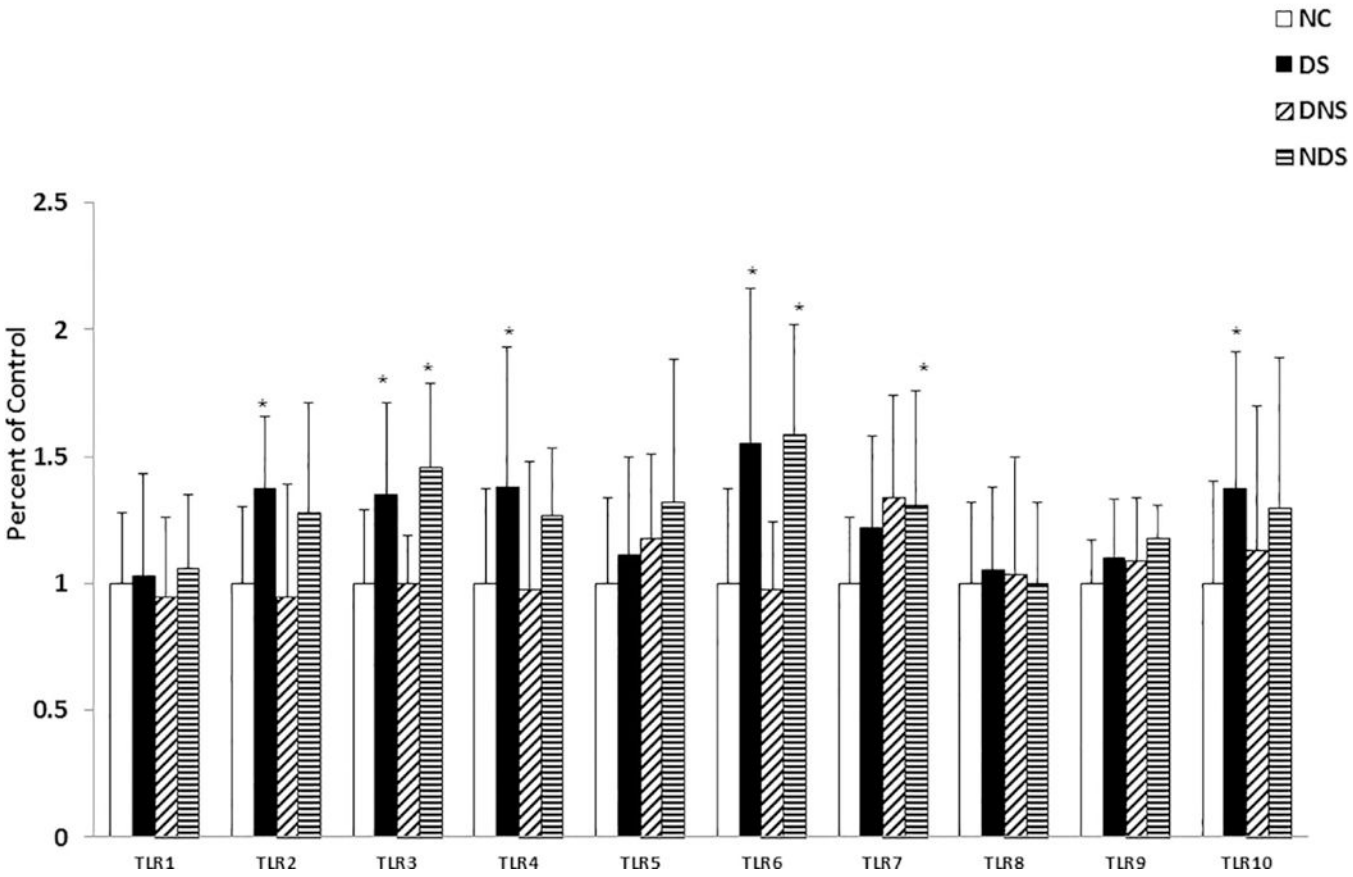

Figure 2.

Mean protein expression levels of TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9 and TLR10 in the PFC of DS (n = 24), DNS (n = 12), NDS (n = 11) and NC (n = 24) subjects.

Values are given as the mean ± SD.

*$p < 0.05$ (compared with NC subjects)

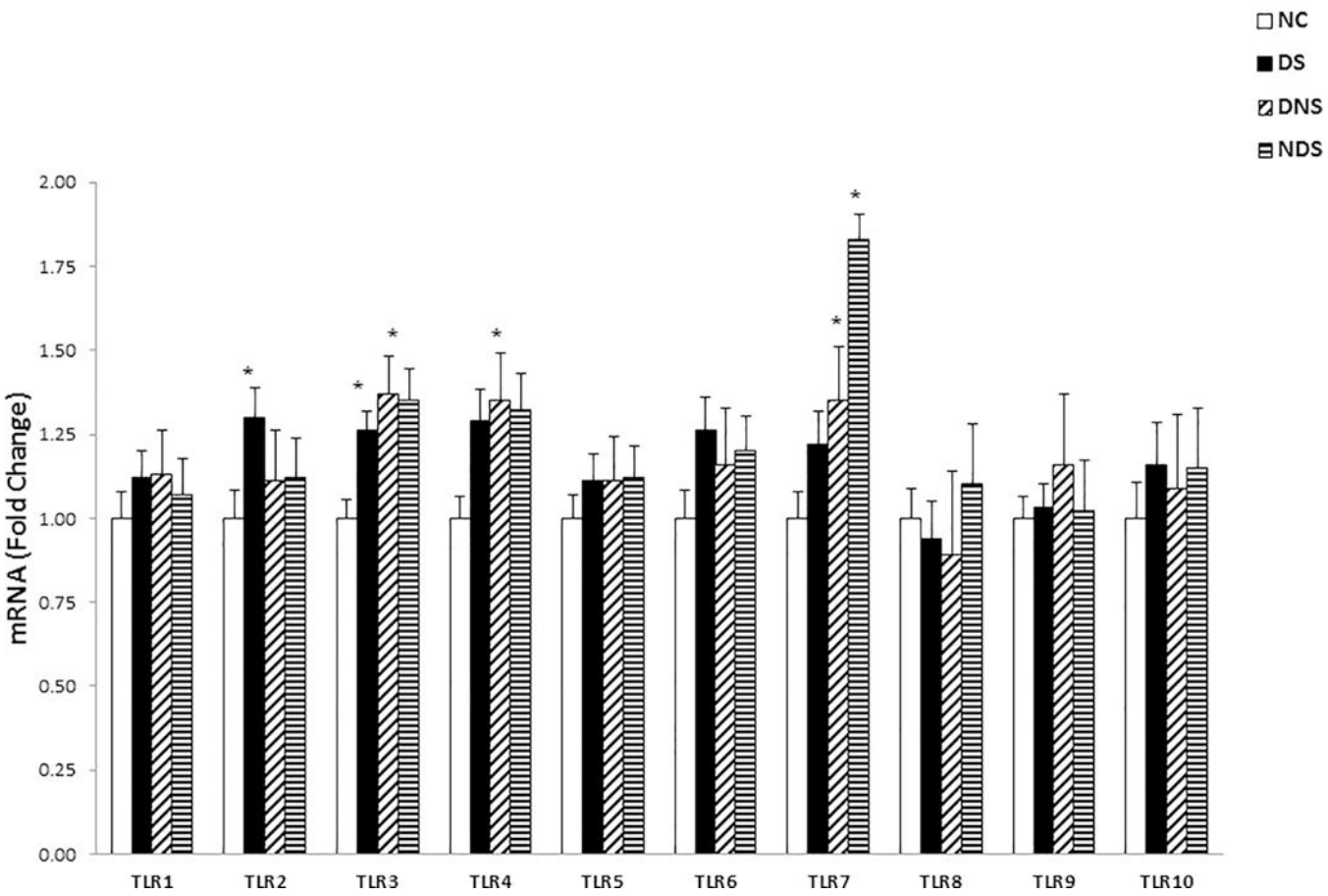

Figure 3.

Mean mRNA expression levels of TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9 and TLR10 in the PFC of DS, DNS, NDS and NC subjects. The data are shown as the fold change in mRNA levels ± S.E.M.

*$p < 0.05$ (compared with NC subjects)

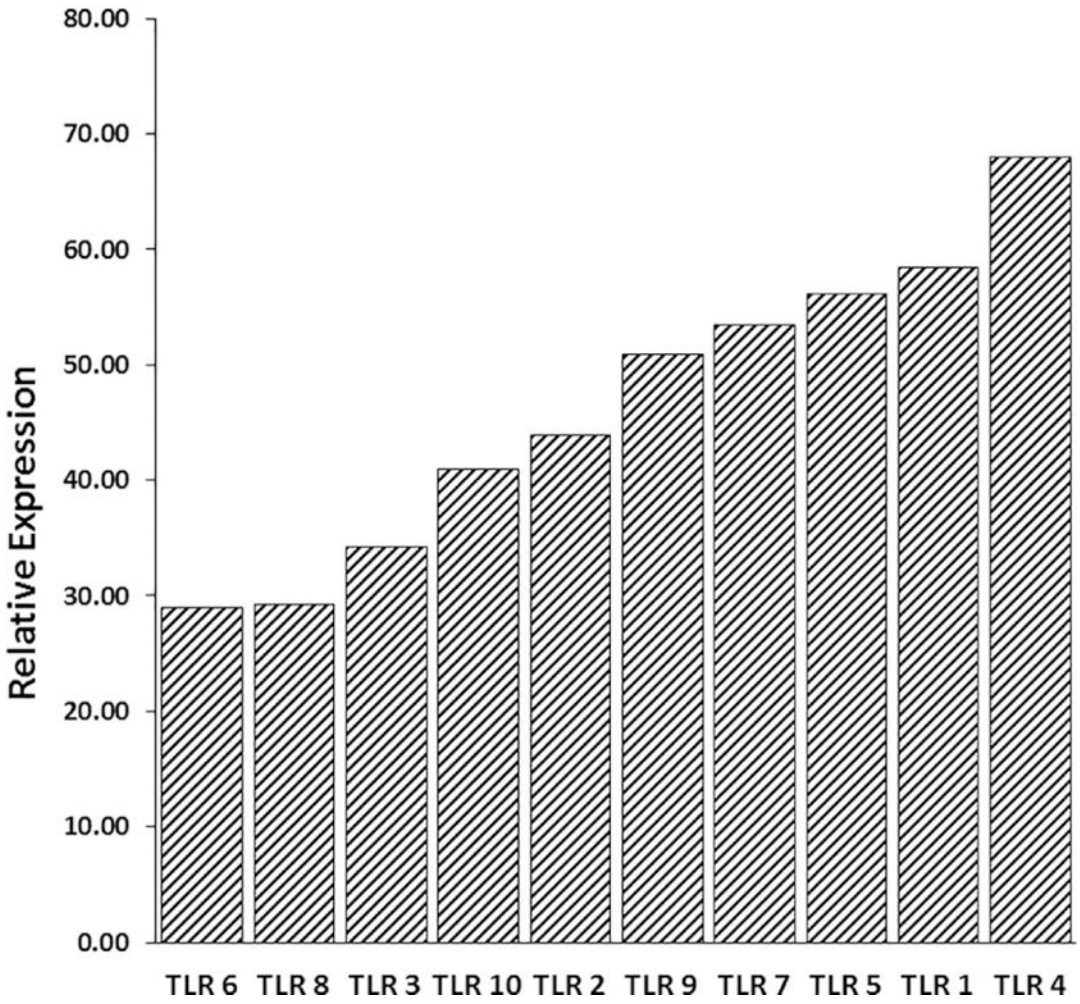

Figure 4.

Relative TLR expression levels in the PFC of NC subjects. Levels are expressed as Log2-transformed mRNA expression level relative to the mean of housekeeping gene ACTB.

Table 1.

TaqMan primers/probes used for qPCR analysis

	TaqMan accession	Probe location (exon boundary)	Assay function
ACTB	Hs99999903_m1	1-1	House Keeping (HK)
GAPDH	Hs99999905_m1	3-3	HK
TRL1	Hs00413978_m1	2-3	target gene
TRL2	Hs01014511_m1	1-2	target gene
TRL5	Hs00152825_m1	3-4	target gene
TRL6	Hs00271977_s1	2-2	target gene
TRL7	Hs00152971_m1	2-3	target gene
TRL8	Hs00607866_mH	1-2	target gene
TRL9	Hs00152973_m1	1-2	target gene
TRL10	Hs01935337_s1	2-2	target gene

Table 2.

Demographic Characteristics of Subjects

	Group	Age (years)	Race	Gender	PMI (hours)	Brain pH	Cause of Death	Psychotropic Drugs (at the time of death)	Psychiatric Diagnosis
Normal Control Subjects [a]									
1.	CONTROL	19	Black	Male	11	6.9	GSW	None	Normal
2.	CONTROL	22	Black	Male	19	6.9	GSW	None	Normal
3.	CONTROL	42	White	Female	23	7.2	Pneumonia	None	Normal
4.	CONTROL	37	Black	Male	5	7.1	ASCVD	None	Normal
5.	CONTROL	31	Black	Male	8	7.2	GSW	None	Normal
6.	CONTROL	46	Black	Male	9	7.1	Multiple injuries	None	Normal
7.	CONTROL	33	White	Male	15	7.0	GSW	None	Normal
8.	CONTROL	48	White	Male	26	6.9	ASCVD	None	Normal
9.	CONTROL	40	White	Female	7	7.0	ASCVD	None	Normal
10.	CONTROL	23	Black	Male	15	6.8	GSW	None	Normal
11.	CONTROL	83	White	Male	20	7.1	ASCVD	None	Normal
12.	CONTROL	65	Black	Female	23	6.9	ASCVD	None	Normal
13.	CONTROL	35	White	Male	24	6.9	Crush injury to abdomen and chest	None	Normal
14.	CONTROL	52	White	Male	30	7.3	ASCVD	None	Normal
15.	CONTROL	37	White	Male	24	7.0	ASCVD	None	Normal
16.	CONTROL	45	White	Male	22	7.3	ASCVD	None	Normal
17.	CONTROL	26	White	Male	12	6.9	Arrhythmia	None	Normal
18.	CONTROL	47	White	Male	10	7.0	ASCVD	None	Normal
19.	CONTROL	31	White	Male	16	7.2	MVA	None	Normal
20.	CONTROL	60	White	Male	15	7.1	Accidental drowning	None	Normal
21.	CONTROL	28	White	Male	13	6.8	Electrocution	None	Normal
22.	CONTROL	45	White	Female	16	6.9	Cardiac arrhythmia	None	Normal
23.	CONTROL	62	White	Male	19	7.0	Cardiac arrest	None	Normal
24.	CONTROL	53	White	Male	15	6.9		None	Normal
Depressed Suicide Subjects [b]									
1.	SUICIDE	27	White	Male	24	7.0	GSW	None	MDD, Ethanol abuse

	Group	Age (years)	Race	Gender	PMI (hours)	Brain pH	Cause of Death	Psychotropic Drugs (at the time of death)	Psychiatric Diagnosis
2.	SUICIDE	44	White	Female	11	7.2	Drug overdose	Nortriptyline	MDD, Ethanol abuse
3.	SUICIDE	36	White	Female	10	7.1	GSW	None	MDD
4.	SUICIDE	24	White	Male	7	7.1	GSW	Ethanol	MDD
5.	SUICIDE	43	White	Male	12	7.0	Drug Overdose	None	MDD, Polysubstance Abuse
6.	SUICIDE	53	White	Male	23	6.9	Jumped from height	None	MDD
7.	SUICIDE	41	White	Female	27	7.1	Drug Overdose	Amitriptyline, Desipramine, Nortriptyline, Ethanol	MDD, Ethanol abuse
8.	SUICIDE	22	Black	Female	16	7.3	Drug overdose	None	MDD
9.	SUICIDE	46	White	Female	21	6.9	Drug overdose	Amitriptyline, Desipramine, Ethanol	MDD
10.	SUICIDE	36	White	Female	18	7.2	GSW	None	MDD
11.	SUICIDE	38	White	Male	24	7.0	Drug overdose & Ethanol overdose	Ethanol	MDD, Ethanol abuse
12.	SUICIDE	46	White	Female	16	6.8	Drug overdose / Nortryptyline Intoxication	Nortriptyline	MDD, Panic disorder with agoraphobia
13.	SUICIDE	23	White	Male	12	7.0	Hanging	Paroxetine	MDD
14.	SUICIDE	18	White	Male	17	6.3	Hanging	None	MDD
15.	SUICIDE	30	White	Male	17	7.1	Hanging	Venlafaxine	MDD
16.	SUICIDE	19	White	Male	18	6.2	CO intoxication	Ethanol, CO	MDD, Ethanol abuse, Polysubstance abuse
17.	SUICIDE	44	White	Female	30	7.2	Drug overdose, Ethanol intoxication	Fluoxetine, Ethanol	MDD, Ethanol abuse, Opioid abuse
18.	SUICIDE	74	White	Female	27	7.0	Venlafaxine overdose	Venlafaxine, Ethanol	MDD, Ethanol abuse
19.	SUICIDE	25	White	Male	14	6.8	Hanging	Ethanol	MDD
20.	SUICIDE	23	White	Male	23	6.9	Hanging	None	MDD
21.	SUICIDE	63	White	Male	19	6.9	Drug overdose, Ethanol intoxication	Ethanol	MDD
22.	SUICIDE	67	White	Male	22	7.0	Venlafaxine overdose	Fluoxetine, Venlafaxine	MDD
23.	SUICIDE	40	White	Female	20	7.0	Drug overdose	Alprazolam	MDD
24.	SUICIDE	53	White	Male	26	7.1	Suicide by stab wound	Sertraline	MDD
Depressed Non-Suicide Subjects [c]									
1.	Non-Suicide depressed	65	White	Male	14	6.9	ASCVD	None	MDD
2.	Non-Suicide depressed	55	Black	Female	8	6.4	ASCVD	Fluoxetine, Ethanol	MDD, Polysubstance abuse
3.	Non-suicide depressed	71	White	Male	4	6.3	ASCVD	Bupropion	MDD

	Group	Age (years)	Race	Gender	PMI (hours)	Brain pH	Cause of Death	Psychotropic Drugs (at the time of death)	Psychiatric Diagnosis
4.	Non-suicide depressed	74	Black	Female	7	6.7	ASCVD	Paroxetine, Thioridazine	MDD
5.	Non-suicide depressed	14	White	Male	11	7.0	MVA	Sertraline	MDD, Polysubstance abuse
6.	Non-suicide depressed	39	White	Male	36	6.8	Fatty Liver	Thioridazine	MDD
7.	Non-suicide depressed	46	Black	Male	20	7.1	Seizure d/o	Fluoxetine, Risperidone	MDD
8.	Non-suicide depressed	59	White	Male	20	7.0	ASCVD	Sertraline	MDD, Ethanol dependence
9.	Non-suicide depressed	46	White	Female	23	6.9	Mixed Drug intoxication	Bupropion, Lamotrigine	MDD, Ethanol abuse, Polysubstance abuse
10.	Non-suicide depressed	29	White	Female	22	6.9	Obesity, Cardiomegal y	Fluoxetine, Norfluoxetine	MDD
11.	Non-suicide depressed	49	White	Male	24	7.1	ASCVD	Desmethylsertrali ne	MDD
12.	Non-suicide depressed	47	White	Female	26	6.5	Diabetic ketoacidosis	Fluoxetine	MDD
Non-Depressed Suicide Subjects[d]									
1.	SUICIDE	34	White	Male	16	6.4	GSW	Ethanol	Ethanol abuse
2.	SUICIDE	21	White	Male	17	6.8	GSW	None	Adjustment disorder mixed
3.	SUICIDE	75	White	Male	18	6.4	GSW	None	Adjustment/cond uct disorder
4.	SUICIDE	87	White	Male	16	6.6	GSW	None	Adjustment/cond uct disorder
5.	SUICIDE	39	White	Male	30	6.7	Asphyxia	Freon, cocaine and metabolites	Cocaine abuse
6.	SUICIDE	30	White	Male	32	6.5	Hanging	Cocaine, Ethanol	Ethanol abuse, Cocaine abuse, Drug abuse
7.	SUICIDE	40	White	Male	26	6.7	GSW	Ethanol	Adjustment disorder
8.	SUICIDE	20	White	Male	32	6.9	Hanging	Ethanol	Ethanol abuse
9.	SUICIDE	71	White	Femal e	24	6.5	Drug OD	None	Adjustment disorder mixed
10.	SUICIDE	24	White	Male	22	6.8	Hanging	None	Schizoaffective disorder
11.	SUICIDE	21	White	Male	23	6.8	Hanging	None	Adjustment/cond uct disorder

ASCVD, atherosclerotic cardiovascular disease; CO, carbon monoxide; GSW, gunshot wound; MDD, major depressive disorder; MVA, motor vehicle accident

[a] Mean ± SD age is 42.08 ± 15.35 years; PMI is 16.54 ± 6.56 hours; brain pH is 7.02 ± 0.15; 7 Black, 17 White; 20 Males, 4 Females

[b] Mean ± SD age is 38.96 ± 15.40 years; PMI is 18.92 ± 6.02 hours; brain pH is 6.96 ± 0.25; 2 Black, 22 White; 14 Males, 10 Females

[c] Mean ± SD age is 49.50 ± 17.18 years; PMI is 17.92 ± 9.32 hours; brain pH is 6.80 ± 0.27*; 3 Black, 9 White; 7 Males, 5 Females

[d] Mean ± SD age is 42.00 ± 24.22 years; PMI is 23.27 ± 6.17* hours; brain pH is 6.65 ± 0.18*; 11 White; 10 Males, 1 Female

* $p < 0.05$ (compared with NC group)

Table 3.

Summary of Patients' Characteristics

	Normal Controls (n = 24)	Depressed Suicide (n = 24)	Depressed Non-Suicide (n = 12)	Non-Depressed Suicide (n = 11)
RIN	6.8 ± 1.2	6.9 ± 1.3	6.8 ± 0.8	7.0 ± 1.2
Age (Years)	42.08 ± 15.35	38.96 ± 15.40	49.50 ± 17.18	42.00 ± 24.22
PMI (hours)	16.54 ± 6.56	18.92 ± 6.02	17.92 ± 9.32	23.27 ± 6.17 *
Brain pH	7.02 ± 0.15	6.96 ± 0.25	6.80 ± 0.27 *	6.65 ± 0.18 *
Race	7 Black 17 White	2 Black 22 White	3 Black 9 White	11 White
Gender	20 Males 4 Females	14 Males 10 Females	7 Males 5 Females	10 Males 1 Felame
Antidepressants (at time of death)	NONE	11 YES 13 NO	11 YES 1 NO	0 YES 11 NO
Ethanol (at time of death)	NONE	9 YES 15 NO	1 YES 11 NO	4 YES 7 NO
Suicide Method	N/A	12 Violent 12 Non-Violent	N/A	9 Violent 2 Non-Violent

Values are mean ± SD

Abbreviations: RIN, RNA integrity number, PMI, postmortem interval

* $p < 0.05$ (compared with NC group)

Table 4.

Toll-like receptors and their ligands

Receptor	Ligand
TLR1	Triacyl lipopeptides, Soluble factors
TLR2	Lipoprotein/lipopeptides, Peptidoglycan, Lipoteichoic acid, Lipoarabinomannan, Phenol-soluble modulin, Glycoinositolphospholipids, Glycolipids, Porins, Atypical lipopolysaccharide, Atypical lipopolysaccharide, Zymosan, Heat-shock protein 70
TLR3	Double-stranded RNA
TLR4	Lipopolysaccharide, Taxol, Fusion protein, Envelope protein, Heat-shock protein 60, Heat-shock protein 70, Type III repeat extra domain A of fibronectin, Oligosaccharides of hyaluronic acid, Polysaccharide fragments of heparan sulphate, Fibrinogen
TLR5	Flagellin
TLR6	Diacyl lipopeptides, Lipoteichoic acid, Zymosan
TLR7	Imidazoquinoline, Loxoribine, Bropirimine, Single-stranded RNA
TLR8	Imidazoquinoline, Single-stranded RNA
TLR9	CpG-containing DNA
TLR10	N.D.
TLR11	N.D.

N.D., not determined; TLR, Toll-like receptor

(Adapted from Akira and Takeda, Nature Reviews 4:499–511, 2004)