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Toll-like receptors in the depressed and suicide brain

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ABSTRACT

Abnormalities of the immune function in depression and suicide are based in part on the observation of increased levels of proinflammatory cytokines in the serum and postmortem brain of depressed and suicidal patients. We have examined if abnormalities of the innate immune receptors, known as Toll-like receptors (TLRs), in the brain are associated with depression and suicide, since the activation of these receptors results in production of cytokines. Of all the TLRs shown to be present in humans, TLR3 and TLR4 appear to be unique and important in brain function. We have determined the protein (by ELISA method) and mRNA expression (using qPCR) of TLR3 and TLR4 in the postmortem brain (dorsolateral prefrontal cortex [DLPFC]) of 22 depressed suicide victims, 11 non-depressed suicide victims, 12 depressed non-suicide subjects and 20 normal control subjects. We found that the mRNA expression of TLR3 and TLR4 was significantly increased in DLPFC of depressed suicide victims and in depressed non-suicide subjects, compared with controls. However, the protein expression of TLR3 and TLR4 was significantly increased in depressed suicide victims, but not in depressed non-suicide subjects compared with controls. The observed abnormalities of proinflammatory cytokines in the brain of suicide victims may be related to an abnormality of TLR3 and TLR4 over-expression. To our knowledge, this is the first study of TLRs in the brain of psychiatric subjects.

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1. Introduction

There are several lines of evidence that suggest abnormalities of the immune system in depression and suicide. This is based on the observation that proinflammatory cytokines, which are released from immune cells as a result of inflammation or stress, are abnormal in the serum of patients with depression [see reviews by Dowlati et al. (2010)]. Other evidence for the involvement of cytokines in depression is derived from studies suggesting that the administration of interferons (IFN), or other cytokines, induces depression in patients with chronic hepatitis C, multiple sclerosis, or some forms of cancer, as reviewed by Capuron et al. (2003).

There is some direct and indirect evidence suggesting a relationship between immune dysregulation and suicide. Steiner et al. (2008) have found increased microgliosis in the postmortem brain of suicide subjects compared with normal control subjects. Lindqvist et al. (2009) found increased levels of interleukin (IL)-6 in cerebrospinal fluid (CSF) of suicide attempters compared with normal control subjects, and Janelidze et al. (2011) observed elevated levels of IL-6 and tumor necrosis factor (TNF)-α and decreased levels of IL-2 in suicide attempters. That cytokine levels may also be abnormal in the brain of suicide victims is substantiated by a report of Pandey et al. (2012) who observed increased protein and mRNA expression levels of IL-1β, IL-6, and TNF-α in the prefrontal cortex of teenage suicide victims compared with normal controls. Shelton et al. (2010), using a microarray method, found that a number of pro- and anti-inflammatory cytokines are upregulated in postmortem brain of subjects with major depressive disorder (MDD). Taken together, these studies suggest abnormalities of the immune function in depression and in suicide in general, and an abnormality of cytokine levels in the postmortem brain of suicide victims in particular.

Although the reasons or mechanism of increased proinflammatory cytokines in the brain of suicide victims as reported by these investigators are not clear, this increase in cytokines may be related to abnormalities of the innate immune receptors, known as Toll-like receptors (TLRs), in the brain (Hanke and Kielian, 2011). There is now increasing evidence to suggest that the central nervous system (CNS) organizes innate immune response during systemic infection and neuronal injury (Crack and Bray, 2007).

Because of the important role TLRs play in neuronal function, and because of their involvement in the production of cytokines and chemokines through MyD88 and MyD88-independent

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pathways, activating nuclear factor *kappa beta* (NFkB) and resulting in the accumulation of chemokines and cytokines (O'Neill, 2006), we examined if abnormalities of TLRs are associated with depression and/or suicide. For that purpose we determined the protein and mRNA expression in dorsolateral prefrontal cortex (DLPFC) obtained from depressed suicide victims, non-depressed suicide victims, depressed non-suicide subjects, and normal controls. Of all the TLRs, we specifically determined the gene and protein expression of TLR3 and TLR4 in this study because (i) TLR3 is the only TLR present in human neurons (Lafon et al., 2006); (ii) it is associated with cognitive function in mice (Okun et al., 2010) and; (iii) both TLR3 and TLR4 are involved in neurite growth and other neuronal functions (Okun et al., 2009). Both TLR3 and TLR4 are also unique compared to other TLRs because of their ability to activate interferon regulatory factor through the MYD88-independent signaling pathway (Okun et al., 2009).

2. Materials and methods

2.1. Subjects and diagnoses

The study was performed in the DLPFC (Brodmann area 9 [BA9]) of 22 depressed suicide victims, 11 non-depressed suicide victims, 12 depressed non-suicide subjects and 20 non-psychiatric control subjects, hereafter referred to as normal control 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.

2.2. Diagnostic method

Subject diagnosis was based on the Structured Clinical Interview for DSM-IV (SCID) (Spitzer et al., 1992). 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 isolation

Total RNA was extracted from 100 mg of tissue using the TRIZOL reagent according to the manufacturer's instructions and treated with DNAse 1 (Invitrogen, USA). The RNA yield was determined by absorbance at 260 nm using NanoDrop®ND-1000 (NanoDrop Technologies, Montchanin, DE, USA). RNA quality was assessed using Agilent Bioanalyzer 2100. All samples had 28S/18S ratios > 1.2 and RNA integrity number (RIN) above 6.6. The mean RIN was 7.2 ± 0.6.

2.3.2. mRNA quantitation

Expression levels of mRNA were determined using a two-step real-time RT-PCR (qPCR) method, which we have previously published (Pandey et al., 2012). Briefly, 1 μg of total RNA was reverse transcribed using MMLV-reverse transcriptase (life technologies) in a final reaction volume of 20 μl qRT-PCR was performed using predesigned Taqman gene expression assays (Applied Biosystems, Foster City, CA) targeting TLR3, Hs 01551078_m1 and TLR4, Hs 00152939_ml along with two housekeeping genes β-actin (ACTB), Hs99999903_m1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Hs99999905_m1. For each primer/probe set, qPCR reaction is carried out using 10 μl of cDNA (diluted 1:10) in 1X TaqMan Universal PCR Master Mix (Applied Biosystems) as per manufacturer's instructions. Each qPCR plate included a "no reverse transcriptase" and "no template" control to eliminate non-specific amplification and each sample is assayed in triplicate.

For qPCR gene expression analysis, raw expression data (C_t) are normalized to the geometric mean of the two housekeeping genes. Outliers were excluded if the normalized (delta C_t) values were greater than 2 standard deviations from the group mean. Relative expression levels, reported as fold change, were determined by $2^{-(\Delta\Delta C_t)}$ method, as described in Applied Biosystems User Bulletin No. 2 (P/N 4303859) and ΔC_t values are used for further statistical analysis.

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).

2.4.1. Quantitation of TLR3 and TLR4 in membrane fraction by Western blot

Immunolabeling of TLR3 and TLR4 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-TLR3 (1:3000 dilution) and polyclonal anti-TLR4 antibody (1:3000 dilution) and subsequently using β-actin monoclonal antibody (1:5000 dilution). The levels of TLR3 and TLR4 proteins were calculated as a ratio of the optical density of the TLR antibody of interest to the optical density of β-actin antibody. TLR3 and TLR4 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, California, USA), and had molecular weights of 117 kDa and 95 kDa, respectively.

2.5. Statistical analysis and effect of confounding variables

We analyzed the data using SAS 9.2 statistical software package. We used ANCOVA (Proc GLM) to jointly compare control subjects, depressed suicide victims, non-depressed suicide victims, and depressed non-suicide subjects adjusting the effects of age, postmortem interval (PMI), and brain pH. Next, we examined the effect of each predictor on the TLR protein and mRNA expressions controlling for other predictors. For example, when the effect of age was examined on TLR (protein or mRNA expressions), we controlled other predictors such as PMI, brain pH, and the group effect. In addition, for multiple comparisons we used *t*-test with Bonferroni Correction to adjust the type I error rates. We also performed a post-hoc *t*-test for each paired comparison separately.

3. Results

3.1. Effect of confounding variables and age on TLR3 and TLR4 expression

The demographic and the clinical characteristics of the suicide victims, depressed subjects, and the normal control subjects are given in Table 1. Our ANCOVA analysis showed that only age has a significant inverse effect on protein or mRNA expression of TLR4 ($F = 4.32$, $p = .04$) suggesting that increasing age is associated with

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