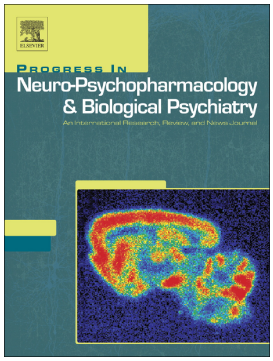

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Differential regulation of the TLR4 signalling pathway in *post-mortem* prefrontal cortex and cerebellum in chronic schizophrenia: Relationship with SP transcription factors.

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KEYWORDS:

Innate immunity, oxidative/nitrosative stress, transcription factors, network interaction analysis, negative symptoms.

ABSTRACT

Alterations in innate immunity may underlie the pathophysiology of schizophrenia (SZ). Toll-like receptor-4 (TLR4) is a master element of innate immunity. The specificity proteins (SPs), transcription factors recently implicated in SZ, are putative regulatory agents of this.

This work was aimed at describing alterations in the TLR4 signalling pathway in postmortem brain prefrontal cortex (PFC) and cerebellum (CB) of 16 chronic SZ patients and 14 controls. The possible association of TLR4 pathway with SP1 and SP4 and SZ negative symptomatology is explored.

In PFC, TLR4/myeloid differentiation factor 88 (MyD88)/inhibitory subunit of nuclear factor kappa B alpha (IκBα) protein levels were lower in SZ patients, while nuclear transcription factor-κB (NFκB) activity, cyclooxygenase-2 (COX-2) expression and the lipid peroxidation index malondialdehyde (MDA) appeared increased. The pattern of changes in CB is opposite, except for COX-2 expression that remained augmented and MDA levels unaltered.

Network interaction analysis showed that TLR4/MyD88/IκBα/NFκB/COX-2 pathway was coupled in PFC and uncoupled in CB. SP4 co-expressed with TLR4 and NFκB in PFC and both SP1 and SP4 co-expressed with NFκB in CB. In PFC, correlation analysis found an inverse relationship between NFκB and negative symptoms.

In summary, we found brain region-specific alterations in the TLR4 signaling pathway in chronic SZ, in which SP transcription factors could participate at different levels. Further studies are required to elucidate the regulatory mechanisms of innate immunity in SZ and its relationship with symptoms.

INTRODUCTION

Schizophrenia (SZ) is a complex disorder with brain connectivity deficits due to alterations in key regions of neuronal circuits. The prefrontal cortex and the cerebellum are part of the cortico-cerebellar-thalamic-cortical circuit, which has been suggested to contribute to symptoms and cognitive impairments in SZ (Andreasen et al. , 1998, Barch, 2014). Alterations in the prefrontal cortex and the cerebellum has been linked to negative symptoms suggesting that both areas could contribute to these symptoms (Hasan et al. , 2017, Li et al. , 2017, McKechanie et al. , 2016, Mittal et al. , 2014, Shaffer et al. , 2015).

An activation of the innate immune system in SZ constitutes an emerging hypothesis of a pathogenic mechanism involved in this disorder (Kirkpatrick and Miller, 2013) that could be altered in brain. The activation of the innate immune system is a non-specific protective response that could become deleterious in pathological, severe or long-lasting conditions (Lampron et al. , 2013). One of the main actors implicated is the family of Toll-like receptors (TLRs), and in particular its most studied member, Toll-like receptor 4 (TLR4). TLRs are pattern recognition receptors highly expressed in peripheral immune cells (Akira et al. , 2006) and also in the Central Nervous System (CNS)(Hanke and Kielian, 2011) that detect both circulating pathogen-associated molecular patterns (Medzhitov, 2001) and a number of endogenous damage-associated molecular signals (Piccinini and Midwood, 2010). Particularly, TLR4 mostly responds to lipopolysaccharide (LPS) from Gram-negative bacteria (Takeuchi and Akira, 2001). Through recruiting adapter proteins, such as the myeloid differentiation factor 88 (MyD88), TLR4 acquires specificity for intracellular signalling (Takeuchi and Akira, 2002). After various consecutive steps in the transduction pathway, the prototypic inflammatory nuclear transcription factor NFκB is activated (Takeuchi and Akira, 2001). NFκB acts on the gene promoters of inducible forms of the enzymes nitric oxide synthase (iNOS) and cyclooxygenase -2 (COX-2), increasing the expression of these genes. The activation of these pro-inflammatory mediators in the brain can produce oxidative/nitrosative stress via lipid peroxidation (Madrigal et al. , 2006). Previous studies report an enhanced peripheral TLR4 expression or response to immune stimuli in SZ (McKernan et al. , 2011, Muller et al. , 2012) and, more recently, an increased expression of TLR4 and MyD88 in *post-mortem* prefrontal cortex (PFC) of SZ patients with high prevalence of suicide has been shown (Garcia-Bueno et al. , 2016). However, the study of the TLR4 pathway in the cerebellum in SZ and in the prefrontal cortex of elderly SZ subjects who died as a result of natural causes has not been explored yet.

Specificity Protein (SP) 1 and 4, which are members of the SP transcription factor family, are recognized as immune system regulators in diverse pathological conditions (Dupuis-Maurin et al. , 2015, Xu et al. , 2012). They have been found to be increased in the hippocampus (Pinacho et al. , 2014). Moreover, SP1 has been found to be reduced in prefrontal cortex in chronic SZ. Reduced levels of SP1 and SP4 protein levels in the cerebellum have been linked to increased severity in negative symptoms in chronic SZ (Pinacho

et al. , 2013). TLR4 gene expression has been reported to be controlled by SP1 (Wasiluk et al. , 2006), suggesting a link between the TLR4 signalling pathway and SP transcription factors. SP1 and SP4 proteins are both members of the SP transcription factor family that bind to gene promoters with almost similar affinities (Suske, 1999), raising the possibility that SP4 protein could also regulate the expression of TLR4. Moreover, it has been reported that SP proteins could be part of the neuronal NFκB binding factor(Mao et al. , 2002), thereby establishing crosstalk between SP transcription factors and NFκB in neurons. Indeed, SP1 is able to bind NFκB sites in promoter regions to modulate neuronal genetic expression (Liu et al. , 2004, Mao et al. , 2006). Together, this growing body of evidence suggests that SP transcriptions factors could be interfering in the TLR4-NFκB pathway in SZ.

The aim of this hypothesis-driven study was to investigate the possible alterations of the TLR4 signalling pathway in prefrontal cortex and cerebellum in chronic SZ and to explore the possible relationship with SP transcription factor proteins and negative symptoms.

MATERIAL AND METHODS

Post-mortem human brain samples

Post-mortem human brain samples from the dorsolateral PFC (Brodmann area 9) and the cerebellum (CB) (lateral cerebellar cortex) of patients with chronic schizophrenia (SZ: PFC, n=15; CB, n=16) and of control subjects with no history of psychiatric episodes (n=14) were obtained from the collection of Neurological Tissue of Sant Joan de Déu (Roca et al. , 2008) and the Hospital Universitari de Bellvitge Brain Bank (Table 1*)*. The demographic and clinical characteristics for these SZ subjects have been previously reported (Pinacho, Villalmanzo, 2013) and are described in detail in Table 1. Specimens, extending from the pial surface to white matter and only including grey matter, were dissected and stored at -80ºC. The study was approved by the Institutional Ethics Committee of Parc Sanitari Sant Joan de Déu. A written informed consent was obtained from each subject. Schizophrenia and control groups were matched by gender, age, post-mortem delay and pH. Table 1 shows the demographic, clinical and tissue-related characteristics of the samples. In the present study, one sample from the schizophrenia group in the prefrontal cortex and one control sample from the cerebellum were not included in the molecular analysis due to the lack of sufficient tissue from that subject (PFC, SZ, n=15) or to undetectable measures (CB, C, n=13). Comparison of demographic and tissue-related measures between groups (control, n=14, SZ, n=15 for PFC and control, n=13, SZ, n=16 for CB) showed similar results (see Table 1). All SZ subjects were institutionalized donors with a long duration of the illness (Table 1) and both control and SZ subjects had no history of neurological episodes. Experienced clinical examiners interviewed each donor *ante-mortem* to confirm SZ diagnosis according to DSM-IV and ICD-10

criteria. Our study includes the following SZ diagnoses: chronic residual schizophrenia (75%, n=12), chronic paranoid SZ (12.5%, n=2), chronic disorganized SZ (6.25%, n=1), chronic catatonic SZ (6.25%, n=1). Moreover, donor subjects were evaluated *ante-mortem* with the Positive and Negative Syndrome Scale (PANSS) and the Clinical Global Impression-Schizophrenia (CGI-SCH) scale with a death to clinical assessment interval shorter than 41 months (Table 1). Five patients were being medicated with first-generation antipsychotics (31.25%), nine were medicated with second-generation antipsychotics (56.25%), of these, eight (57.14%) were medicated with a combination of two or more antipsychotics of both first and second generation, and two were antipsychotic-free (12.5%). In particular, the following antipsychotics were present: haloperidol (n=4), levomepromazine (n=2), clozapine (n=8), quetiapine (n=4), clotiapine (n=1), risperidone (n=1), sulpiride (n=1), amisulpride (n=1), zolpidem (n=1). To control for the possible effect of antipsychotic treatment on the molecular measure, the last mean daily chlorpromazine equivalent dose was calculated for the antipsychotic treatment of each patient based on the electronic records of last drug prescriptions administered up to death (Table 1) as previously described (Gardner et al., 2010). This so-called *chlorpromazine equivalent dose* provides a comparable standardised measure for each patient that allows for the comparison of different doses of antipsychotics among patients treated with different combinations of highly heterogeneous antipsychotic compounds. Given the heterogeneity of the treatment regimens of the patients in this study a statistical comparison of different treatment regimens would not be feasible.

Preparation of nuclear and cytosolic extracts from tissue samples

A modified procedure based on the method of Schreiber et al. (1989) was used: 100mg of tissue was homogenized in 300 µL buffer (10 mmol/L *N*-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (pH 7.9), 1 mmol/L EDTA, 1 mmol/L EGTA, 10 mmol/L KCl, 1 mmol/L dithiothreitol, 0.5 mmol/L phenylmethylsulfonyl fluoride, 0.1 mg/mL aprotinin, 1 mg/mL leupeptin, 1 mg/mL Na-*p*-tosyll-lysine-chloromethyl ketone, 5 mmol/L NaF, 1 mmol/L NaVO4, 0.5 mol/L sucrose, and 10 mmol/L Na2MoO4). After 15 min, Nonidet P-40 (Roche, Mannheim, Germany) was added to a concentration of 0.5%. The tubes were gently vortexed for 15 secs, and nuclei were collected by centrifugation at 8000*g* for 5 min. The supernatants were considered to be the cytosolic fraction. The pellets were resuspended in 100 µL buffer supplemented with 20% glycerol and 0.4 mol/L KCl and gently shaken for 30 min at 4ºC. Nuclear protein extracts were obtained by centrifugation at 13000*g* for 5 min, and aliquots of the supernatant were stored at -80ºC. All the fractionation steps were carried out at 4ºC.

Western blot analysis

TLR4, MyD88, iNOS and COX-2 protein levels were analysed in homogenized PFC and CB samples. In the case of NF-κB, nuclear extracts were analysed, while for the inhibitory subunit of NFκB, IκBα, cytosolic extracts were used. SP1 and SP4 protein levels in the PFC and CB were previously measured in the same subjects (Pinacho, Villalmanzo, 2013).

To determine the expression levels of TLR4, MyD88, iNOS and COX-2, 100mg of PFC and CB tissue samples were homogenized by sonication in phosphate-buffered saline (PBS) mixed with a protease inhibitor cocktail (Complete®, Roche Farma, Spain) (pH=7); followed by centrifugation at 12000*g* for 10 min at 4ºC.

After adjusting the protein levels in the resultant supernatants, homogenates were mixed with Laemmli sample buffer (BioRad, Hercules, CA, USA) and 15 µg were loaded onto an electrophoresis gel. Samples from each SZ subject and the corresponding matched control were always loaded on the same gel and run in parallel. Each experiment was repeated at least twice. Two control brain samples were loaded in all the experiments as a reference value to control the inter-experimental variability. Next, the membranes were blocked in 10 mM tris-buffered saline containing 0.1% Tween-20 and 5% skimmed milk or bovine serum albumin (BSA). Then the membranes were incubated with specific primary antibodies from Santa Cruz Biotechnology (CA, USA) against: iNOS (rabbit polyclonal antibody raised against a peptide mapping to the amino terminus of iNOS of human origin at a dilution of 1:1000 in TBS-Tween) (sc-651); COX-2 (goat polyclonal antibody raised against a peptide mapping to the C-terminus of COX-2 of mouse origin at a dilution of 1:750 in 5% BSA in TBS-Tween) (sc-1747); NFκB p65 subunit (rabbit polyclonal antibody raised against an epitope mapping within the C-terminus of NFκB p65 of human origin at a dilution of 1:500 in BSA 2%) (sc-372); IκBα (rabbit polyclonal antibody raised against a peptide mapping to the C-terminus of IκBα of human origin at a dilution of 1:1000 in BSA 2%) (sc-371); TLR-4 (rabbit polyclonal antibody raised against an epitope corresponding to amino acids 242-321 mapping to an internal region of TLR-4 of human origin (sc-10741). They were also incubated with the specific primary antibody from Abcam © (Cambridge, UK) against: MyD88 (rabbit polyclonal antibody raised against amino acids 279-296 of MyD88 of human origin at a dilution of 1:1000 in BSA 2%) (ab-2064). The respective blocking peptides were used (when available) to check antibody specificity. After washing with 10 mM tris-buffered saline containing 0.1% Tween-20, the membranes were incubated with the respective horseradish peroxidase-conjugated secondary antibodies for 90 min at room temperature. Blots were imaged using an Odyssey® Fc System (Li-COR Biosciences) and were quantified by densitometry (NIH ImageJ® software). All densitometries are expressed in arbitrary units of optical density (OD). In all Western blot analyses, β-actin (mouse monoclonal antibody at a dilution of 1:15000 from Clone AC-15; Sigma, Spain) was used as a loading control, except for the NFκB p65 subunit, in

which case the loading control was GAPDH (mouse monoclonal antibody at a dilution of 1:2000 (G8795; Sigma) (blots shown in the respective figures).

SP1 and SP4 protein levels in the PFC and CB were previously measured in the same subjects (Pinacho, Villalmanzo, 2013). Briefly, 100mg of human brain samples were homogenized on ice in a glass douncer with NP40 lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, 2 mM EDTA, 2 mM EGTA, 10 mM Na-β-glycerophosphate, 5mM sodium pyrophosphate, 1mM Na_3VO_4, 1% β-mercaptoethanol, 1mM phenylmethylsulphonylfluoride, 50mM NaF, 25 mM N-ethylmaleimide and protease inhibitor cocktail (Roche Diagnostics))as described (Pinacho et al. , 2011)). 50 µg of randomized human protein lysates were analysed in a blind manner and resolved by SDS/PAGE electrophoresis and immunoblotted with polyclonal antibody against SP4 (Santa Cruz Technology, sc-645, 1/250), SP3 (Santa Cruz Technology, sc-644, 1/250) or SP1 (Millipore,07-645, 1µg/ml), and monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Millipore, MAB374, 1/500,000). Densitometric quantification was performed using Quantity One software (BioRad). Values were normalized to GAPDH and to a reference sample.

Lipid peroxidation

Lipid peroxidation was measured using a modification of the method by Das & Ratty (1987), in which thiobarbituric acid reactive substances (TBARS), predominantly malondialdehyde (MDA) produced as a secondary product, were quantified using the 2-thiobarbituric acid (TBA) colour reaction. Brain tissue was homogenized in 10 volumes (w/v) of sodium phosphate buffer (pH 7.4). Assays contained tissue homogenate, trichloroacetic acid (40% w/v), HCl (5 M) and TBA (2% w/v). The samples were heated to 90ºC and kept at that temperature for 15 min, then centrifuged at 12000*g* for 10 min. The MDA-TBA adduct (pink chromogen) of the supernatant was measured spectrophotometrically (at 532 nm) and the malondialdehyde concentration calculated using a standard curve prepared with malondialdehyde tetrabutylammonium salt. The results are expressed as nmol/mg protein.

Network interaction analysis

Interaction between the main elements of the TLR4 pathway and its relationship with SP transcription factors were evaluated using a co-expression analysis adapted from a previously described study in schizophrenia (Hirayama-Kurogi et al. , 2017). This co-expression analysis for characterising a pathway in a biological substrate is based on the assumption that proteins functionally related co-expressed (Stuart et al. , 2003). Briefly, among all samples studied, only the samples with the highest (percentile 75^{th}) and lowest (percentile 25^{th}) protein expression levels of the upstream member of the pathway were selected

for analysis, starting from TLR4 (i.e. High-TLR4 group or Low-TLR4 group). Correlation analyses with the downstream member of the pathway were performed. Differences in the protein expression levels of the downstream member between the group with samples with the highest protein levels and the group with the lowest protein levels were evaluated. Significant correlations in this co-expression analysis indicate that the interaction distance between these two proteins is short in this network (Hirayama-Kurogi, Takizawa, 2017). We indicated that the pathway was "coupled" when all the downstream members of the pathway correlate with the upstream member, reflecting the proximity of the members in the pathway. The term "uncoupled pathway" was used when no significant correlations were detected between the downstream proteins and the upstream member in the pathway, indicating a higher distance between the elements of the pathway.

Statistical analysis

Quantitative values for protein or activity were tested for a Gaussian distribution using the D'Agostino & Pearson omnibus normality test and compared using Student's unpaired t-test or Mann-Whitney test according to the distribution of each variable. Outliers were detected where indicated using Pierce's criterion (as simplified by Gould) (Ross, 2003) for non-parametric variables and Grubbs test for parametric variables. Spearman or Pearson correlation analyses were carried out to detect association of the molecular measures with potential confounding factors (age, *post-mortem* delay, pH, daily chlorpromazine equivalent dose (CPZd) and duration of illness). In case of finding a significant association with a potential confounder factor, a multiple linear regression model with a stepwise forward procedure was used to adjust for the co-variable where indicated. The False Discovery Rate (FDR) with the Benjamini and Hochberg method (Benjamini and Hochberg, 1995) was computed for all the p values resulting from the comparisons with symptoms in each brain region. The FRD threshold was set to 0.1. Statistical analyses were performed with GraphPad Prism version 5.00 and SPSS 24. All statistical tests were two-tailed and significance level was set to 0.05.

RESULTS

1.- TLR4 proinflammatory-oxidant pathway in *post-mortem* brain PFC samples of subjects with chronic SZ and matched controls.

We first characterised the protein expression of members of the TLR4 proinflammatory-oxidant pathway in the PFC in SZ by western blot analysis. We found that TLR4 and MyD88 protein expression was significantly decreased by 0.74- and 0.75-fold in the PFC of SZ subjects compared to controls (TLR4: Student t=3.038, df=27, p value=0.0052. MyD88: t=2.052, df=27, p value=0.0500, Figs. 1A, B). Although cytosolic IκBα expression was significantly decreased by 0.59-fold in SZ (Mann-Whitney U=28.00, p value=0.0014; Fig. 1C), there were no significant changes in the nuclear protein levels of NFκB (Fold change=1.19, t=1.636, df=27, p value=0.1134; Fig. 1D); and NFκB activity showed a small trend to be increased in SZ subjects (Fold change= 1.10, t=1.938, df=25, p value=0.064; Fig. E). iNOS expression was not changed in SZ subjects compared to control individuals (Fold change=1.04, t=0.4816, df=25, p value=0.6343; Fig 1F), while COX-2 protein levels were significantly increased by 1.25-fold in the SZ group (t=2.657, df=26, p value=0.0133, Fig 1G). Finally, levels of MDA were significantly increased by 2.21-fold in the SZ group (t=3.195, df=26, p value=0.0036; Fig 1H).

We used bivariate analyses to evaluate the influence of potential confounders in the significant changes detected in the PFC. MyD88 associated with PMD (r Spearman=0.403, p value=0.0302), COX-2 with age (r Pearson=-0.382, p value=0.0446, and MDA with pH (r Spearman=-0.387, p value=0.0420) in the PFC in the SZ-control comparison (Table 2). Linear regression analysis revealed that the changes of COX-2 protein levels and MDA in SZ subjects remained significant after adjusting for age (β =0.501, p=0.002, adjusted R^2= 0.941), and pH (β =0.876, p<0.001, adjusted R^2= 0.759) respectively. However, the influence of PMD on MyD88 levels in SZ was not ruled out (β =0.167, p=0.272, adjusted R^2= 0.842). We further explored the possible influence of the duration of the illness and the antipsychotic treatments on the TLR4 signalling pathway components. For this purpose, we first converted the last daily dose of the antipsychotic regime prescribed to each patient to equivalents of chlorpromazine as described by Gardner and co-workers (Gardner et al., 2010). No associations were found between TLR4 signalling elements and the significant altered proteins in SZ and the last daily chlorpromazine equivalent dose (Table 2).

We then explored whether the elements of the TLR4 signalling pathway in the PFC correlate with the severity of negative symptoms measured by the Positive and Negative Syndrome Schizophrenia (PANSS) scale and the Clinical Global Impression-Schizophrenia Scale (CGI-SCH) (Table 3). NFκB activity and protein levels inversely correlated with the severity of negative symptoms in SZ patients (PANSS: activity (r

Pearson=-0.650, p=0.0119); protein levels (r Pearson=-0.554, p=0.0322); CGI-SCH: activity (r Pearson=-0.620, p=0.0181); protein levels (r Pearson=-0.648, p=0.009)). After correction of significance by applying a False Discovery Rate of 0.1, NFκB activity remained significant for negative symptoms in the PANSS scale (q value=0.0952, p value threshold=0.0125) and both NFκB activity and protein levels for negative symptoms in the CGI-SCH scale (activity: q value=0.0965, p value threshold=0.0188; protein levels: q value=0.0952; p value threshold=0.0063) (Table 3).

2.- TLR4 proinflammatory-oxidant pathway in *post-mortem* CB samples of subjects with chronic SZ and matched controls.

We found a significant 1.38- and 1.17-fold increase in the protein expression of TLR4 and MyD88, respectively, in the CB of SZ subjects compared to matched controls (TRL4: Student t=2.250, df=24, p value=0.0339. MyD88: t=2.137, df=24, p value=0.0430; Figs. 2A, B). IκBα cytoplasmic expression was significantly higher in SZ (Fold change=1.51, t=2.262, df=26, p value=0.0323; Fig. 2C) while NFκB expression in nuclear extracts did not differ between the groups studied (Fold change=0.79, t=1.613, df=24, p value=0.1199; Fig 2D). In contrast, NFκB activity in nuclei was significantly reduced by 0.80-fold in SZ subjects (t=2.817, df=25, p value=0.0093; Fig 2E). As observed for the PFC, iNOS expression was not changed (Fold change=1.05, Mann-Whitney U=84.00, p value=0.7524; Fig 2F), while COX-2 protein levels were significantly increased by 1.23-fold in the SZ group (t=2.130, df=24, p value=0.0436; Fig 2G). There were no significant changes in MDA levels between the groups (Fold change=1.25, t=1.245, df=26, p value=0.2242; Fig 2H).

The analysis of confounding variables did not reveal any significant associations with potential confounders (Table 2). We then explored the effect of duration of the illness and antipsychotic treatment (expressed in the form of chlorpromazine equivalent dose, CPZd) on our molecular measures. We found a significant inverse correlation between CPZd and TLR4 protein levels in the CB in SZ (r Pearson=-0.568, p value=0.0340; (Table 2).

We then explored if elements of the TLR4 signalling pathway in the CB correlate with the severity of negative symptoms measured by the PANSS and CGI-SCH scales (Table 3). We found significant correlations of NFκB and IκBα levels with negative symptoms in the CGI-SCH scale (NFκB: r Pearson = -0.569, p=0.0269; IκB: r Pearson =0.529, p value=0.0425) (Table 3). However, after applying a False Discovery Rate of 0.1, these correlations were no longer significant (Table 3).

3- Network interaction analysis of TLR4 proinflammatory-oxidant pathway and of SP1 and SP4 proteins.

To explore the possible interaction of SP1 and SP4 with the TLR4 proinflammatory-oxidant pathway in the PFC and CB we used a co-expression analysis (Figures 3 and 4). For these analyses, we used the protein levels of SP4 and SP1 determined by immunoblot in the same samples of this cohort in a previous study (Pinacho, Villalmanzo, 2013) which showed the following results in SZ group compared to healthy controls: in the PFC, SP1 (Fold change=0.75, t=1.861 df=28, p=0.0367) and SP4 (Fold change=0.76, t=1.303 df=28, p=0.2033), and in the CB, SP1 (Fold change=1.151, Mann-Whitney U=88, p=0.4715) and SP4 (Fold change =0.8127, t=0.680, df=28, p=0.5021). Our co-expression analysis shows that SP4 expression significantly correlates with TLR4 (r Pearson=0.527, p value=0.053) and NFκB activity (r Pearson=0.554, p value=0.050) in the selected samples, while SP1 shows no any association with TLR4 (r Spearman=0.204, p value=0.483; Figure 3B), suggesting that the interaction distance between SP4 and TLR4 and between SP4 and NFκB activity is short in the PFC. In addition, comparison between the groups showed reduced TLR4 levels in individuals with lower levels of SP4 compared to individuals with higher levels of SP4 in the PFC (SP4-TLR4: Mann-Whitney U=8.00, p value=0.0379; Figure 3C), while no differences were detected for the SP1 groups (SP1-TLR4: Mann-Whitney U=13.00, p value=0.1649; Figure 3D). NFκB activity was also significantly reduced in individuals with lower SP4 protein levels (SP4-NFκB activity: Mann-Whitney U=6.00, p value=0.0350; Figure 3E). Moreover, interactions between protein pairs showed significant associations between TLR4-MyD88 (r Spearman=0.600, p value=0.023), MyD88-IκBα (r Pearson=0.573, p value=0.032), IκBα-NFκB activity (r Pearson=-0.591, p value=0.043) and NFκB activity-COX-2 (r Pearson=0.572, p value=0.041), but not between the pair NFκB activity-iNOS (r Pearson=0.323, p value=0.281; Figure 3B). There were also significant protein differences in some of the downstream molecules between high- and low-upstream protein groups (TLR4-MyD88 (Mann-Whitney U=4.00, p value=0.0070), MyD88-IκBα (U=9.00, p value=0.0289), IκBα-NFκB activity (U=0.00, p value=0.0006) and NFκB activity-COX-2 (U=1.00, p value=0.0051), Figure 3F-J). These results indicate that SP4 is acting upstream and downstream of TLR4 with the coupled TLR4-MyD88-IκBα-NFκB-COX-2 pathway, which was altered in the PFC in our experimental setting.

In the CB, the expression levels of SP4 and SP1 significantly correlated with NFκB (SP4: r Spearman=0.769, p value=0.003; SP1: r Pearson=0.824, p value=0.017) and iNOS (SP4: r Spearman=0.818, p value=0.002; SP1: r Spearman=0.850, p value=0.0005) in the selected samples (Figure 4A and B), suggesting that the interaction distance between SP proteins and NFκB is short in the CB. In addition, comparison between groups showed that NFκB and iNOS protein levels were reduced in individuals with lower levels of SP proteins (SP1-NFκB: Mann-Whitney U=6.00, p value=0.0350; SP4-NFκB: Mann-Whitney U=1.00, p value=0.0043, SP1-iNOS: Mann-Whitney U=3.00, p value=0.0152, SP4-iNOS: Mann-Whitney U=2.00, p value=0.0173; Figure 4C-F). iNOS, but not COX-2, protein levels were also significantly reduced in individuals with lower NFκB protein levels (NFκB-iNOS: Mann-Whitney U=5.00, p value=0.0076; NFκB-COX-2: Mann-Whitney U=26.00, p value=0.9546; Figure 4G and H). However, interactions between protein pairs showed

TLR4-iNOS (r Spearman=0.608, p value=0.036) and TLR4-COX-2 (r Pearson=0.697, p value=0.012) associations (Figure 4B). There were only significant protein differences in downstream molecules between high and low-upstream protein groups in the case of groups of high- and low- TLR4 protein levels when evaluating iNOS and COX-2 levels (TLR4-iNOS: U Mann-Whitney =3.00, p value=0.0152; TLR4-COX-2: U Mann-Whitney =5.00, p value=0.0411; Figure 4I-L). These results indicate that SP1 and SP4 proteins are interacting downstream of TLR4 with the uncoupled and altered TLR4-MyD88-IκBα-NFκB pathway in the CB in SZ through their interaction with NFκB.

DISCUSSION

In the present study, we have found differential alterations in the TLR4 signalling pathway in the PFC and CB *post-mortem* samples of patients with chronic SZ. Network interaction analysis showed that the elements of the TLR4 signalling pathway present greater proximity in the cell signalling network in the PFC than in the CB. In addition, interactions between protein pairs showed that SP4 is acting upstream and downstream of TLR4 with the coupled TLR4-MyD88-IκBα-NFκB-COX-2 pathway in the PFC and in the cerebellum, and that SP1 and SP4 proteins are interacting downstream of TLR4 with the uncoupled TLR4-MyD88-IκBα-NFκB pathway. Finally, correlation analysis found an inverse relationship between NFκB and negative symptoms in the PFC.

Our results provide new evidence to add to the role of TLR4 in SZ pathophysiology. The prevailing knowledge proposes the TLR system not only as an important regulator of the characteristic low-grade inflammatory process of SZ (i.e. enhanced peripheral TLRs responses have been demonstrated in 40 patients with SZ, producing a massive release of IL-6 or TNF-α in stimulated whole blood with TLR2 and 4 agonists (McKernan, Dennison, 2011), as well as increased TLR-4 expression in the monocytes of 31 SZ patients compared to matched controls (Muller, Wagner, 2012), but also as a key element in neuroplasticity (neurogenesis, axonal growth, and synaptic remodelling) in the healthy and diseased brain, influencing cognition-related processes such as memory, learning and even mood regulation (Garcia Bueno et al. , 2016, Hanke and Kielian, 2011, Okun et al. , 2011).

In this vein, the differential regulation of the TLR4 signalling pathway in *post-mortem* brain PFC and CB found here illustrates the complexity of the inflammatory response in pathological conditions and, as a consequence, the difficulty of designing therapeutic strategies based on the modulation of inflammation (Leza et al. , 2015). Variability in the state, degree and evolution of the inflammatory response in pathological conditions between different brain areas has been previously suggested (Martin-Hernandez et al. , 2016), and could be related to the number and type of microglial cells, the proximity to circumventricular organs, the degree of Oxygen/Nitrogen free radicals generated in neuronal activity, etc. In

this regard, *in vitro* cultures of murine neurons from the PFC and CB showed that cortical cells are much more susceptible to NFκB-induced inflammation than cerebellar neurons (Young et al. , 2012).

The extent and duration of TLR activation during innate immune responses has been suggested to be mediated by targeting TLRs to proteosomal degradation through ubiquitination (Chuang and Ulevitch, 2004). MyD88 and IκBα, as other proteins of TLR signalling, are also controlled by ubiquitin posttranslational modification and subsequent degradation (Heaton et al. , 2016). In this study, we found that TLR4, MyD88 and IκBα protein levels were increased in the CB and decreased in the PFC in SZ, suggesting that ubiquitin-mediated mechanisms that control TLR4 signalling could be deregulated in an opposite way in both brain areas. In this regard, evidence of altered ubiquitination patterns in *post-mortem* brain in elderly chronic SZ has been described (Rubio et al. , 2013).

In our study, although TLR4-MyD88-IκBα-NFκB elements are proximal in the cell signalling network in the PFC, decreased levels of TLR4 and MyD88 did not correlate with an increase in NFκB activity as has been classically described (Takeuchi and Akira, 2001). One possible explanation may be that increased NFκB activity may downregulate TLR4-MyD88 expression as a compensatory mechanism against excessive inflammation. In the CB, we found that the TLR4, MyD88, IκBα, and NFκB elements are not close, suggesting that the reduction of NFκB activity does not only depend on the increase in TLR4 levels in these patients. Further studies are needed to explore in depth the mechanisms involved in TLR4-MyD88 and IκBα-NFκB reciprocal regulation in both brain areas in elderly chronic schizophrenia subjects.

We recently demonstrated the activation of the TLR4 pro-inflammatory pathway in *post-mortem* PFC samples of patients diagnosed with chronic SZ (Garcia-Bueno, Gasso, 2016). However, the opposite results found here compared to our previous study could be due to differences in the clinical characteristics of the cohorts studied and methodological considerations. Firstly, there are considerable differences in PMD between both cohorts. Secondly, the present study only used samples from male subjects. The age (older) and cause of death (exclusively natural causes in the present study against the 50% of cases of suicide in the previous one) are also different, as well as the duration of the illness (more chronicity in the present study).

One relevant issue is the elucidation of the origin and putative regulatory pathways implicated in the innate immune alterations observed so far in psychotic disorders (Garcia-Bueno et al., 2016). SP1 and SP4 proteins appear to be an excellent set of candidates to regulate innate immunity. Thus, our correlation analysis between SPs and the TLR4 signalling pathway reflects a complex relationship that could be interpreted in different ways. In the PFC, SP4 seems to be very closely related to TLR4 and NFκB activity (see Figures 3A-B and S1), suggesting that SP4 could be facilitating TLR4 and NFκB activity. In our conditions, SP1 is not closely related to TLR4 and NFκB activity in the PFC, but other authors have found that SP1 regulates the transcriptional activity of the murine TLR4 promoter (Wasiluk, McCulloch, 2006). More recently, it has been shown that SP1 is capable of upregulating TLR4 expression through the binding of SP1 to the

hypomethylated TLR4 promoter in gastric cells (Kim et al. , 2016). In the case of the CB, our analysis indicates that SP1 and SP4 are related to NFκB activity and iNOS expression level. This differential interaction upstream and downstream of TLR4 could explain the different state of the TLR4 proinflammatory-oxidant pathway in both brain regions studied; however, further functional interaction studies are required to precisely delineate the role of SPs in the regulation of the TLR4 signalling pathway.

The opposite relationship could also take place; SP1 expression may be triggered by NFκB activity after stimulation of human peripheral blood mononuclear cells with the viral mimetic Poly(I:C) (Doyle et al. , 2013). However, it has been recently demonstrated that LPS downregulates SP1 activity by activating the NFκB pathway in endotoxemic mice (Ye et al. , 2015). Thus, SP proteins could be upregulated in a homeostatic response to increase TLR4-NFκB activity. This response could be related to the ability of SP1 to upregulate the levels of NFκB blocking anti-inflammatory cytokine IL-10 in monocytes (Norkina et al. , 2007). A recent *in silico* approach also incorporates a further degree of complexity, suggesting that SP1 mediates crosstalk between the family of interferon regulatory factors and NFκB pathways orchestrating TLRs-dependent antiviral responses (Iwanaszko and Kimmel, 2015).

To our knowledge, our study is the first to show a direct correlation between SP4 and TLR4 proteins in PFC samples of SZ subjects. Indeed, this putative relationship should be further confirmed in future studies, as well as its biological significance for psychotic disease, in the context of the emerging role of SP4 in the regulation of dopaminergic, glutamatergic and GABAergic neurotransmission (Nair et al. , 2016, Priya et al. , 2013). In this regard, both SP4 and TLR4 have also been reported to be involved in neurodevelopmental processes (Ramos et al. , 2007, Rolls et al. , 2007). Thus, the identified interaction of TLR4 and SP4 in the PFC could impact on neuronal circuitry development and functioning leading to connectivity defects found in patients with SZ.

Taking into account the dual nature (pro *vs* anti-inflammatory profile) of certain inflammatory mediators, their putative correlations with positive and negative symptoms in psychotic diseases (Edwards et al. , 2016, Khandaker et al. , 2015) are sometimes difficult to explain. In our study, after applying the False Discovery Rate, we found an inverse relationship between NFκB and negative symptoms in the PFC, and a direct relationship of TLR4 and iNOS with positive symptoms in the CB. NFκB is a master regulator of acute stress-induced inflammatory response but is also an essential regulatory element of cell survival, controlling key processes such as neuroprotection, neuronal transmission and plasticity, and long-term memory (Kaltschmidt and Kaltschmidt, 2015). It is worth mentioning that in our network interaction analysis in both brain regions MDA levels are not closely related to NFκB-iNOS-COX-2 levels as expected. This could suggest an involvement of alternative oxidative-nitrosative stress mechanisms not related to NFκB activation (i.e. dysregulation of antioxidant endogenous systems). Further studies should evaluate whether increased NFκB activity is a predominant homeostatic response specific to this brain area.

We found a significant inverse correlation between CPZd and TLR4 protein levels in the CB within the SZ group that deserves further investigation. Similarly, the administration of paliperidone to mice subjected to chronic stress blocked the induced activation of the TLR4 signalling pathway in brain (MacDowell et al. , 2014). More recently, a chronic treatment with paliperidone blocked the activation of TLR-3 signalling pathway elicited by a maternal immune activation (with the viral mimetic poly(I:C)) mouse model of SZ (MacDowell et al. , 2017) and abnormal expression of TLRs in monocytes and T-cells can be detected in the earliest stage of SZ, which is in part normalized by antipsychotics at least in the case of TLR4 in monocytes (Keri et al. , 2017).

Several limitations of our study should be acknowledged. First, our cohort consisted of males only, which does not represent the real population of the disease. Further studies are needed to investigate the likely gender differences in this pro-inflammatory pathway. Second, the study included elderly individuals due to the type of sample available for the study. Third, although symptoms and in particular, negative symptoms, may be stable measures in chronic patients (Reichenberg et al. , 2005), small variations in the clinical scores up to death may still slightly affect the associations we report here, thus they may need to be taken with caution. Fourth, most of the patients were on antipsychotic treatments, which could influence the results. However, we have tested the possibility of an effect of antipsychotic treatment on our findings by means of analysing the effect of the last chlorpromazine equivalent dose in our findings. This allowed us to identify an effect of antipsychotic treatment on TLR4 levels in the CB as discussed above. Fifth, the co-expression analysis is a prediction of how members of TLR4 pathway are functionally related among them and with SP proteins in each brain area and thus, future experimental interaction analyses are needed to confirm these results. Further studies in a younger and larger cohort with equal representation of both genders and, if possible, drug naïve patients would be of interest.

Despite these limitations, we report the existence of brain area-specific alterations in the TLR4 signalling pathway in *post-mortem* cerebral tissue from chronic SZ patients. In addition, we have found correlations between certain members of this inflammatory pathway with SPs, which have recently been implicated in SZ pathophysiology. Moreover, we show specific correlations between certain inflammatory-related mediators and negative symptomatology. Further studies are required to analyse the temporal and mechanistic complexity of the inflammatory response in different brain regions, and its relationship with putative regulatory pathways and with symptoms in SZ. Over the last few years, alterations in innate immunity/inflammation have been found in major neuropsychiatric diseases (Garcia Bueno, Caso, 2016), SZ included (Venkatasubramanian and Debnath, 2013), but, as our differential results suggest, significant scientific effort is still needed to corroborate the therapeutic potential of a proper pharmacological modulation of TLRs signalling pathways.

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Disclosure

The authors declare no conflict of interest.

REFERENCE LIST

Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell. 2006;124:783-801.
Andreasen NC, Paradiso S, O'Leary DS. "Cognitive dysmetria" as an integrative theory of schizophrenia: a dysfunction in cortical-subcortical-cerebellar circuitry? Schizophr Bull. 1998;24:203-18.
Barch DM. Cerebellar-thalamic connectivity in schizophrenia. Schizophr Bull. 2014;40:1200-3.
Benjamini Y, Hochberg Y. Controlling the false discovery rate: A practical and powerful approach to multiple testing. J Roy Stat Soc Ser B. 1995;57:289–300.
Chuang TH, Ulevitch RJ. Triad3A, an E3 ubiquitin-protein ligase regulating Toll-like receptors. Nat Immunol. 2004;5:495-502.
Doyle SL, Shirey KA, McGettrick AF, Kenny EF, Carpenter S, Caffrey BE, et al. Nuclear factor kappaB2 p52 protein has a role in antiviral immunity through IkappaB kinase epsilon-dependent induction of Sp1 protein and interleukin 15. J Biol Chem. 2013;288:25066-75.
Dupuis-Maurin V, Brinza L, Baguet J, Plantamura E, Schicklin S, Chambion S, et al. Overexpression of the transcription factor Sp1 activates the OAS-RNAse L-RIG-I pathway. PLoS One. 2015;10:e0118551.
Edwards AC, Bigdeli TB, Docherty AR, Bacanu S, Lee D, de Candia TR, et al. Meta-analysis of Positive and Negative Symptoms Reveals Schizophrenia Modifier Genes. Schizophr Bull. 2016;42:279-87.
Garcia-Bueno B, Gasso P, MacDowell KS, Callado LF, Mas S, Bernardo M, et al. Evidence of activation of the Toll-like receptor-4 proinflammatory pathway in patients with schizophrenia. J Psychiatry Neurosci. 2016;41:E46-55.
Garcia Bueno B, Caso JR, Madrigal JL, Leza JC. Innate immune receptor Toll-like receptor 4 signalling in neuropsychiatric diseases. Neurosci Biobehav Rev. 2016;64:134-47.
Gardner DM, Murphy AL, O'Donnell H, Centorrino F, Baldessarini RJ. International consensus study of antipsychotic dosing. Am J Psychiatry. 2010;167:686-93.
Hanke ML, Kielian T. Toll-like receptors in health and disease in the brain: mechanisms and therapeutic potential. Clin Sci (Lond). 2011;121:367-87.
Hasan A, Wobrock T, Guse B, Langguth B, Landgrebe M, Eichhammer P, et al. Structural brain changes are associated with response of negative symptoms to prefrontal repetitive transcranial magnetic stimulation in patients with schizophrenia. Mol Psychiatry. 2017;22:857-64.
Heaton SM, Borg NA, Dixit VM. Ubiquitin in the activation and attenuation of innate antiviral immunity. J Exp Med. 2016;213:1-13.
Hirayama-Kurogi M, Takizawa Y, Kunii Y, Matsumoto J, Wada A, Hino M, et al. Downregulation of GNA13-ERK network in prefrontal cortex of schizophrenia brain identified by combined focused and targeted quantitative proteomics. J Proteomics. 2017;158:31-42.
Iwanaszko M, Kimmel M. NF-kappaB and IRF pathways: cross-regulation on target genes promoter level. BMC Genomics. 2015;16:307.
Kaltschmidt B, Kaltschmidt C. NF-KappaB in Long-Term Memory and Structural Plasticity in the Adult Mammalian Brain. Front Mol Neurosci. 2015;8:69.
Keri S, Szabo C, Kelemen O. Antipsychotics influence Toll-like receptor (TLR) expression and its relationship with cognitive functions in schizophrenia. Brain Behav Immun. 2017;62:256-64.
Khandaker GM, Cousins L, Deakin J, Lennox BR, Yolken R, Jones PB. Inflammation and immunity in schizophrenia: implications for pathophysiology and treatment. Lancet Psychiatry. 2015;2:258-70.

Kim TW, Lee SJ, Oh BM, Lee H, Uhm TG, Min JK, et al. Epigenetic modification of TLR4 promotes activation of NF-kappaB by regulating methyl-CpG-binding domain protein 2 and Sp1 in gastric cancer. Oncotarget. 2016;7:4195-209.
Kirkpatrick B, Miller BJ. Inflammation and schizophrenia. Schizophr Bull. 2013;39:1174-9.
Lampron A, Elali A, Rivest S. Innate immunity in the CNS: redefining the relationship between the CNS and Its environment. Neuron. 2013;78:214-32.
Leza JC, Garcia-Bueno B, Bioque M, Arango C, Parellada M, Do K, et al. Inflammation in schizophrenia: A question of balance. Neurosci Biobehav Rev. 2015;55:612-26.
Li Z, Lei W, Deng W, Zheng Z, Li M, Ma X, et al. Aberrant spontaneous neural activity and correlation with evoked-brain potentials in first-episode, treatment-naive patients with deficit and non-deficit schizophrenia. Psychiatry Res. 2017;261:9-19.
Liu A, Hoffman PW, Lu W, Bai G. NF-kappaB site interacts with Sp factors and up-regulates the NR1 promoter during neuronal differentiation. J Biol Chem. 2004;279:17449-58.
MacDowell KS, Caso JR, Martin-Hernandez D, Madrigal JL, Leza JC, Garcia-Bueno B. Paliperidone prevents brain toll-like receptor 4 pathway activation and neuroinflammation in rat models of acute and chronic restraint stress. Int J Neuropsychopharmacol. 2014;18.
MacDowell KS, Munarriz-Cuezva E, Caso JR, Madrigal JL, Zabala A, Meana JJ, et al. Paliperidone reverts Toll-like receptor 3 signaling pathway activation and cognitive deficits in a maternal immune activation mouse model of schizophrenia. Neuropharmacology. 2017;116:196-207.
Madrigal JL, Garcia-Bueno B, Caso JR, Perez-Nievas BG, Leza JC. Stress-induced oxidative changes in brain. CNS Neurol Disord Drug Targets. 2006;5:561-8.
Mao X, Moerman-Herzog AM, Wang W, Barger SW. Differential transcriptional control of the superoxide dismutase-2 kappaB element in neurons and astrocytes. J Biol Chem. 2006;281:35863-72.
Mao X, Moerman AM, Barger SW. Neuronal kappa B-binding factors consist of Sp1-related proteins. Functional implications for autoregulation of N-methyl-D-aspartate receptor-1 expression. J Biol Chem. 2002;277:44911-9.
Martin-Hernandez D, Bris AG, MacDowell KS, Garcia-Bueno B, Madrigal JL, Leza JC, et al. Modulation of the antioxidant nuclear factor (erythroid 2-derived)-like 2 pathway by antidepressants in rats. Neuropharmacology. 2016;103:79-91.
McKechanie AG, Moorhead TW, Stanfield AC, Whalley HC, Johnstone EC, Lawrie SM, et al. Negative symptoms and longitudinal grey matter tissue loss in adolescents at risk of psychosis: preliminary findings from a 6-year follow-up study. Br J Psychiatry. 2016;208:565-70.
McKernan DP, Dennison U, Gaszner G, Cryan JF, Dinan TG. Enhanced peripheral toll-like receptor responses in psychosis: further evidence of a pro-inflammatory phenotype. Transl Psychiatry. 2011;1:e36.
Medzhitov R. Toll-like receptors and innate immunity. Nat Rev Immunol. 2001;1:135-45.
Mittal VA, Dean DJ, Bernard JA, Orr JM, Pelletier-Baldelli A, Carol EE, et al. Neurological soft signs predict abnormal cerebellar-thalamic tract development and negative symptoms in adolescents at high risk for psychosis: a longitudinal perspective. Schizophr Bull. 2014;40:1204-15.
Muller N, Wagner JK, Krause D, Weidinger E, Wildenauer A, Obermeier M, et al. Impaired monocyte activation in schizophrenia. Psychiatry Res. 2012;198:341-6.
Nair B, Johar K, Priya A, Wong-Riley MT. Specificity protein 4 (Sp4) transcriptionally regulates inhibitory GABAergic receptors in neurons. Biochim Biophys Acta. 2016;1863:1-9.
Norkina O, Dolganiuc A, Shapiro T, Kodys K, Mandrekar P, Szabo G. Acute alcohol activates STAT3, AP-1, and Sp-1 transcription factors via the family of Src kinases to promote IL-10 production in human monocytes. J Leukoc Biol. 2007;82:752-62.
Okun E, Griffioen KJ, Mattson MP. Toll-like receptor signaling in neural plasticity and disease. Trends Neurosci. 2011;34:269-81.
Piccinini AM, Midwood KS. DAMPening inflammation by modulating TLR signalling. Mediators Inflamm. 2010;2010.
Pinacho R, Valdizan EM, Pilar-Cuellar F, Prades R, Tarrago T, Haro JM, et al. Increased SP4 and SP1 transcription factor expression in the postmortem hippocampus of chronic schizophrenia. J Psychiatr Res. 2014;58:189-96.

Pinacho R, Villalmanzo N, Lalonde J, Haro JM, Meana JJ, Gill G, et al. The transcription factor SP4 is reduced in postmortem cerebellum of bipolar disorder subjects: control by depolarization and lithium. Bipolar Disord. 2011;13:474-85.
Pinacho R, Villalmanzo N, Roca M, Iniesta R, Monje A, Haro JM, et al. Analysis of Sp transcription factors in the postmortem brain of chronic schizophrenia: a pilot study of relationship to negative symptoms. J Psychiatr Res. 2013;47:926-34.
Priya A, Johar K, Wong-Riley MT. Specificity protein 4 functionally regulates the transcription of NMDA receptor subunits GluN1, GluN2A, and GluN2B. Biochim Biophys Acta. 2013;1833:2745-56.
Ramos B, Gaudilliere B, Bonni A, Gill G. Transcription factor Sp4 regulates dendritic patterning during cerebellar maturation. Proc Natl Acad Sci U S A. 2007;104:9882-7.
Reichenberg A, Rieckmann N, Harvey PD. Stability in schizophrenia symptoms over time: findings from the Mount Sinai Pilgrim Psychiatric Center Longitudinal Study. J Abnorm Psychol. 2005;114:363-72.
Roca M, Escanilla A, Monje A, Baño V, Planchat L, Costa J, et al. Banco de tejidos neurológicos de Sant Joan de Déu-Serveis de Salut Mental para la investigación de las enfermendades mentales. La importancia de un programa de donación en vida. Psiquiatría Biológica. 2008;15:73-9.
Rolls A, Shechter R, London A, Ziv Y, Ronen A, Levy R, et al. Toll-like receptors modulate adult hippocampal neurogenesis. Nat Cell Biol. 2007;9:1081-8.
Ross SM. Peirce's criterion for the elimination of suspect experimental data. J Eng Technol. 2003;20:38-41.
Rubio MD, Wood K, Haroutunian V, Meador-Woodruff JH. Dysfunction of the ubiquitin proteasome and ubiquitin-like systems in schizophrenia. Neuropsychopharmacology. 2013;38:1910-20.
Shaffer JJ, Peterson MJ, McMahon MA, Bizzell J, Calhoun V, van Erp TG, et al. Neural Correlates of Schizophrenia Negative Symptoms: Distinct Subtypes Impact Dissociable Brain Circuits. Mol Neuropsychiatry. 2015;1:191-200.
Stuart JM, Segal E, Koller D, Kim SK. A gene-coexpression network for global discovery of conserved genetic modules. Science. 2003;302:249-55.
Suske G. The Sp-family of transcription factors. Gene. 1999;238:291-300.
Takeuchi O, Akira S. Toll-like receptors; their physiological role and signal transduction system. Int Immunopharmacol. 2001;1:625-35.
Takeuchi O, Akira S. MyD88 as a bottle neck in Toll/IL-1 signaling. Curr Top Microbiol Immunol. 2002;270:155-67.
Venkatasubramanian G, Debnath M. The TRIPS (Toll-like receptors in immuno-inflammatory pathogenesis) Hypothesis: a novel postulate to understand schizophrenia. Prog Neuropsychopharmacol Biol Psychiatry. 2013;44:301-11.
Wasiluk KR, McCulloch KA, Banton KL, Dunn DL. Sp1 elements regulate transcriptional activity within the murine Toll-like receptor 4 promoter. Surg Infect (Larchmt). 2006;7:489-99.
Xu HG, Jin R, Ren W, Zou L, Wang Y, Zhou GP. Transcription factors Sp1 and Sp3 regulate basal transcription of the human IRF-3 gene. Biochimie. 2012;94:1390-7.
Ye X, Liu H, Gong YS, Liu SF. LPS Down-Regulates Specificity Protein 1 Activity by Activating NF-kappaB Pathway in Endotoxemic Mice. PLoS One. 2015;10:e0130317.
Young AM, Campbell EC, Lynch S, Dunn MH, Powis SJ, Suckling J. Regional susceptibility to TNF-alpha induction of murine brain inflammation via classical IKK/NF-kappaB signalling. PLoS One. 2012;7:e39049.

FIGURE CAPTIONS

Figure 1. TLR4 pathway in *post-mortem* human brain prefrontal cortex of SZ subjects and matched controls.

TLR4 (A), MyD88 (B), IκBα (C), NFκB (D) protein levels; Activity of NFκB (E) and protein levels of iNOS (F) and COX2 (G); MDA levels (H) in PFC of schizophrenic subjects (SZ) and matched controls (C). The densitometric data of the respective bands of interest are normalized with respect to β-actin/GAPDH (lower band). *p<0.05; **p<0.01 vs. control (C). [a]Unpaired Student's t-test analysis. [b]Mann-Witney test. Data represent the mean ± standard error of the mean.

TLR4: toll-like receptor 4; MyD88: myeloid differentiation factor 88; NFκB: nuclear factor κB; IκBα: inhibitory protein κBα; iNOS: inducible nitric oxide synthase; COX-2: cyclooxygenase-2; MDA: malondialdehyde; GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

Figure 2. TLR4 pathway in *post-mortem* human cerebellum of schizophrenic subjects and matched controls.

TLR4 (A), MyD88 (B), IκBα (C), NFκB (D) protein levels; Activity of NFκB (E) and protein levels of iNOS (F) and COX2 (G); MDA levels (H) in cerebellum of schizophrenic subjects (SZ) and matched controls (C). The densitometric data of the respective bands of interest are normalized with respect to β-actin/GAPDH (lower band). *p<0.05; **p<0.01 vs. control (C). [a]Unpaired Student's t-test analysis. [b]Mann-Witney test. Data represent the mean ± standard error of the mean.

TLR4: toll-like receptor 4; MyD88: myeloid differentiation factor 88; NFκB: nuclear factor κB; IκBα: inhibitory protein κBα; iNOS: inducible nitric oxide synthase; COX-2: cyclooxygenase-2; MDA: malondialdehyde; GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

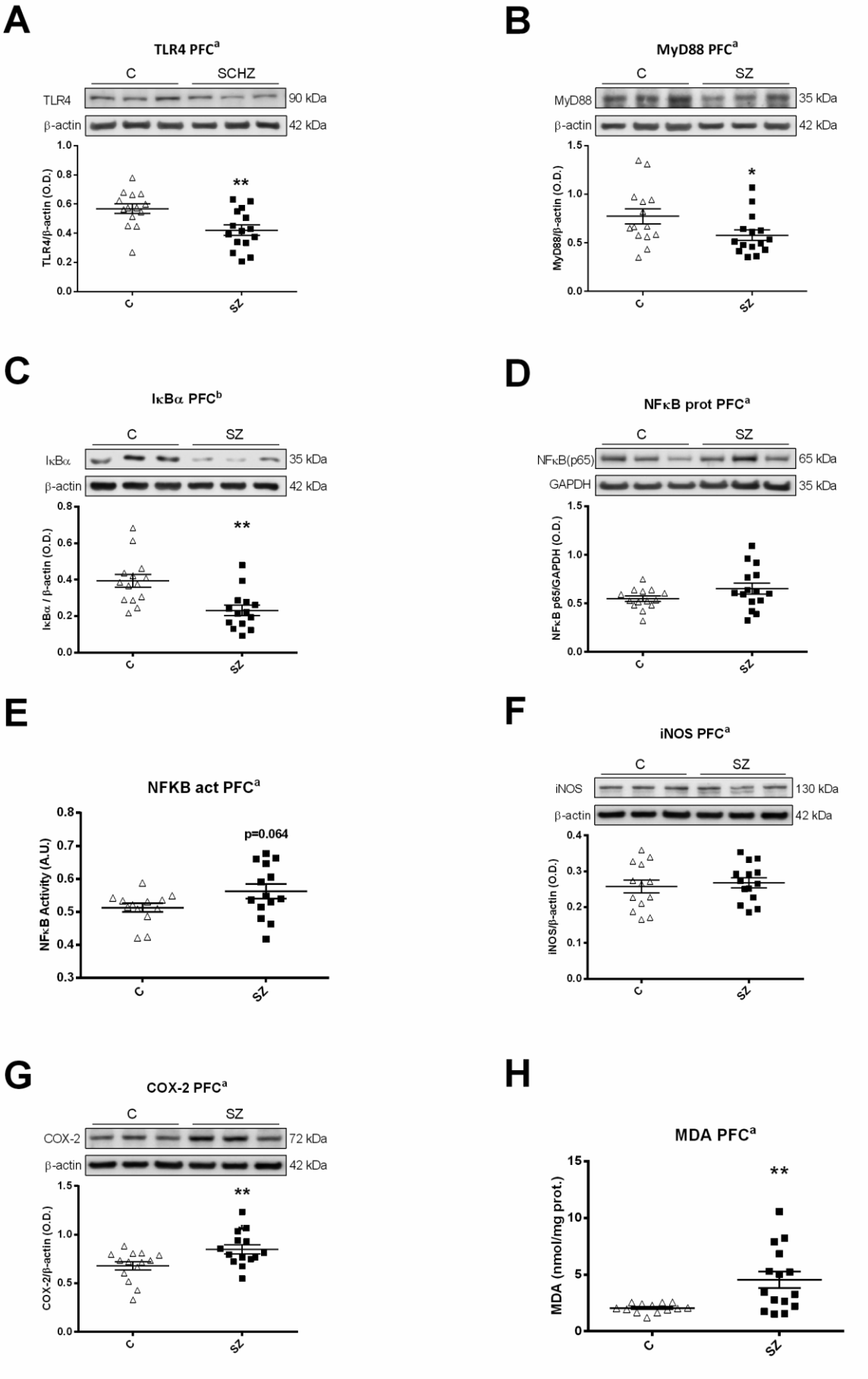
Figure 3. Co-expression analysis of proteins of TLR4 proinflammatory/oxidant pathway and of SP4 and SP1 proteins in *postmortem* prefrontal cortex.

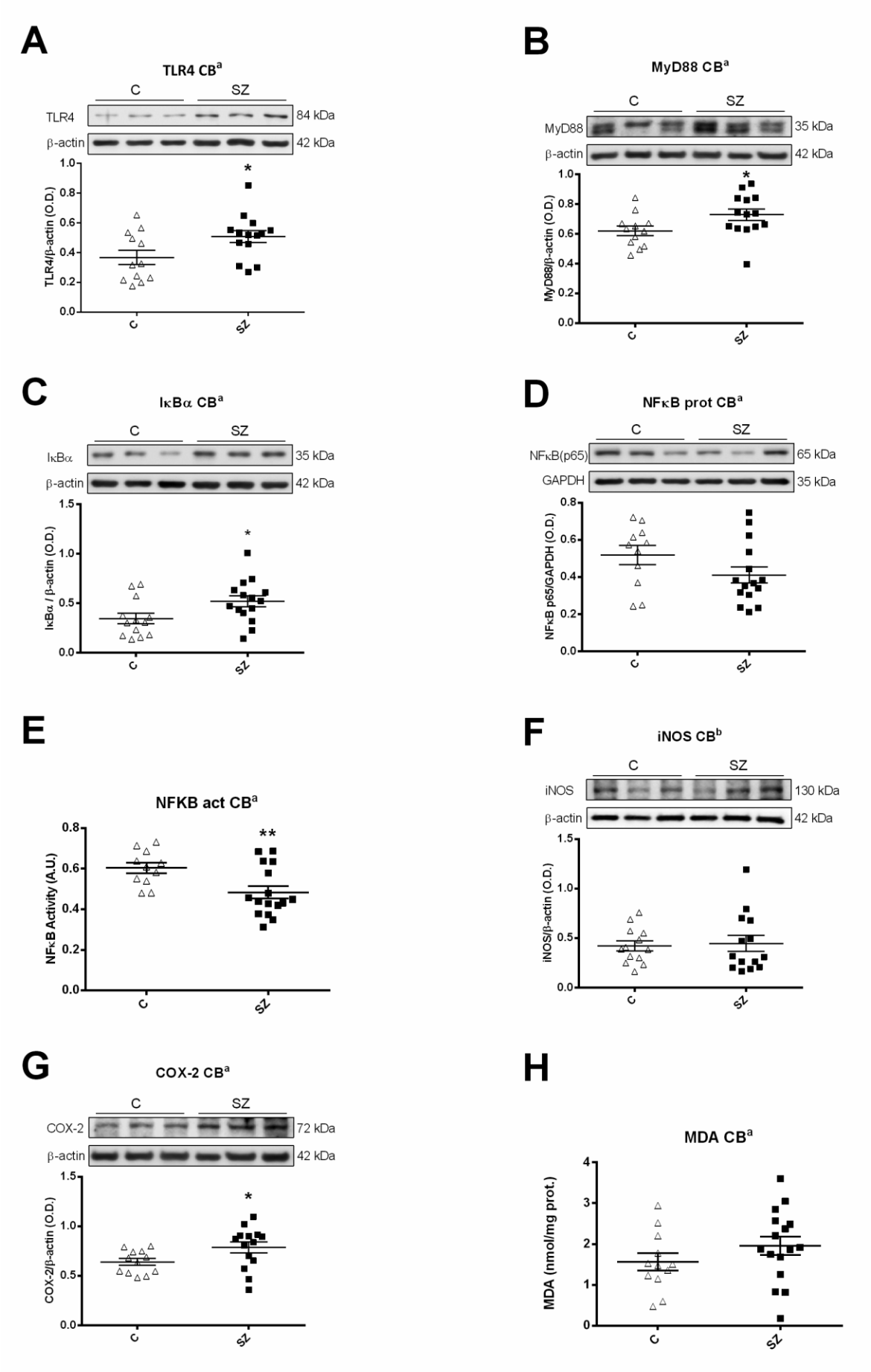
(A) Network model of TLR4 proinflammatory/oxidant pathway. The arrows show signal transduction between upstream and downstream elements. Filled circle indicates a significantly correlated or changed protein in interaction analysis (p<0.05). (B and C-J) Only the samples with the highest and lowest protein

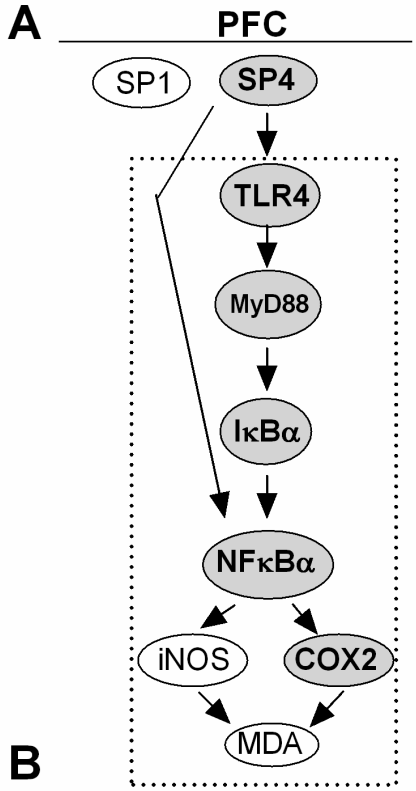
expression levels of the upstream member of the pathway were selected for the analysis. (B) Correlation analyses with the downstream member of the pathway were performed (n=13-15) using Pearson or Spearman analysis. Significant p values are labelled in bold. (C-J) Differences in the protein levels of the downstream element between the High- and Low- group were evaluated. *p<0.05; **p<0.01. Mann-Whitney test. Data represent the mean ± standard error of the mean.

Figure 4. Co-expression analysis of proteins of TLR4 proinflammatory/oxidant pathway and of SP4 and SP1 proteins in *postmortem* cerebellum.

(A) Network model of TLR4 proinflammatory/oxidant pathway. Filled circle indicates a significantly correlated or changed protein in the co-expression analysis (p<0.05). (B and C-L) Analysis was performed as described in Figure 2. (B) Correlation analyses with the downstream member of the pathway were performed (n=13-15) using Pearson or Spearman analysis. Significant p values are labelled in bold. (C-L) Differences in the protein levels of the downstream element between the High- and Low- group were evaluated. *p<0.05; **p<0.01. Mann-Whitney test. Data represent the mean ± standard error of the mean.


A
TLR4 PFC[a]
C
SCHZ
TLR4
90 kDa
β-actin
42 kDa
TLR4/β-actin (O.D.)
**
C
SZ
B
MyD88 PFC[a]
C
SZ
MyD88
35 kDa
β-actin
42 kDa
MyD88/β-actin (O.D.)
*
C
SZ
C
IκBα PFC[b]
C
SZ
IκBα
35 kDa
β-actin
42 kDa
IκBα / β-actin (O.D.)
**
C
SZ
D
NFκB prot PFC[a]
C
SZ
NFκB(p65)
65 kDa
GAPDH
35 kDa
NFκB p65/GAPDH (O.D.)
C
SZ
E
NFKB act PFC[a]
p=0.064
NFκB Activity (A.U.)
C
SZ
F
iNOS PFC[a]
C
SZ
iNOS
130 kDa
β-actin
42 kDa
iNOS/β-actin (O.D.)
C
SZ
G
COX-2 PFC[a]
C
SZ
COX-2
72 kDa
β-actin
42 kDa
COX-2/β-actin (O.D.)
**
C
SZ
H
MDA PFC[a]
MDA (nmol/mg prot.)
**
C
SZ

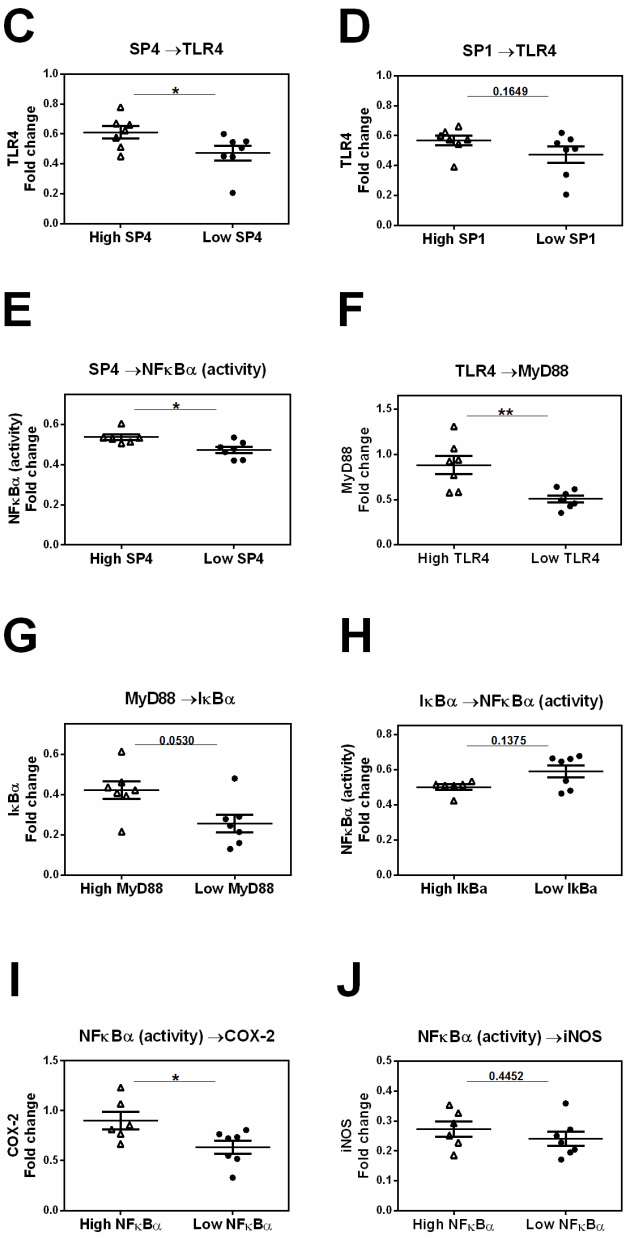


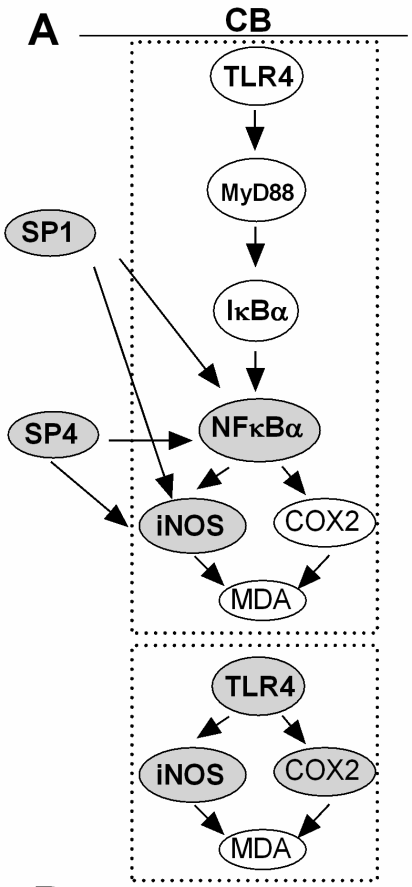


Relation	R	p value
SP1-TLR4#	0.204	0.483
SP4-TLR4	**0.527**	**0.053**
SP4-NFκB activity	**0.554**	**0.050**
TLR4-MyD88#	**0.600**	**0.023**
MyD88-IκBα	**0.573**	**0.032**
IkBa-NFκB activity	**-0.591**	**0.043**
NFκB activity-COX2	**0.572**	**0.041**
NFκB activity-iNOS	0.323	0.281
COX2-MDA	0.116	0.706

R, Pearson coeficient

#Spearman test applied





B

Relation	R	p value
SP1- NFκB	**0.824**	**0.017**
SP4- NFκB #	**0.769**	**0.003**
SP1-iNOS#	**0.850**	**0.0005**
SP4-iNOS#	**0.818**	**0.002**
NFκB -iNOS#	**0.643**	**0.010**
NFκB -COX2#	0.211	0.451
iNOS-MDA	0.109	0.712
COX2-MDA	0.446	0.169
TLR4-MyD88	0.431	0.162
MyD88- IκBα	0.182	0.572
TLR4-COX2	**0.697**	**0.012**
TLR4-iNOS#	**0.608**	**0.036**

R, Pearson coeficient
#Spearman test applied

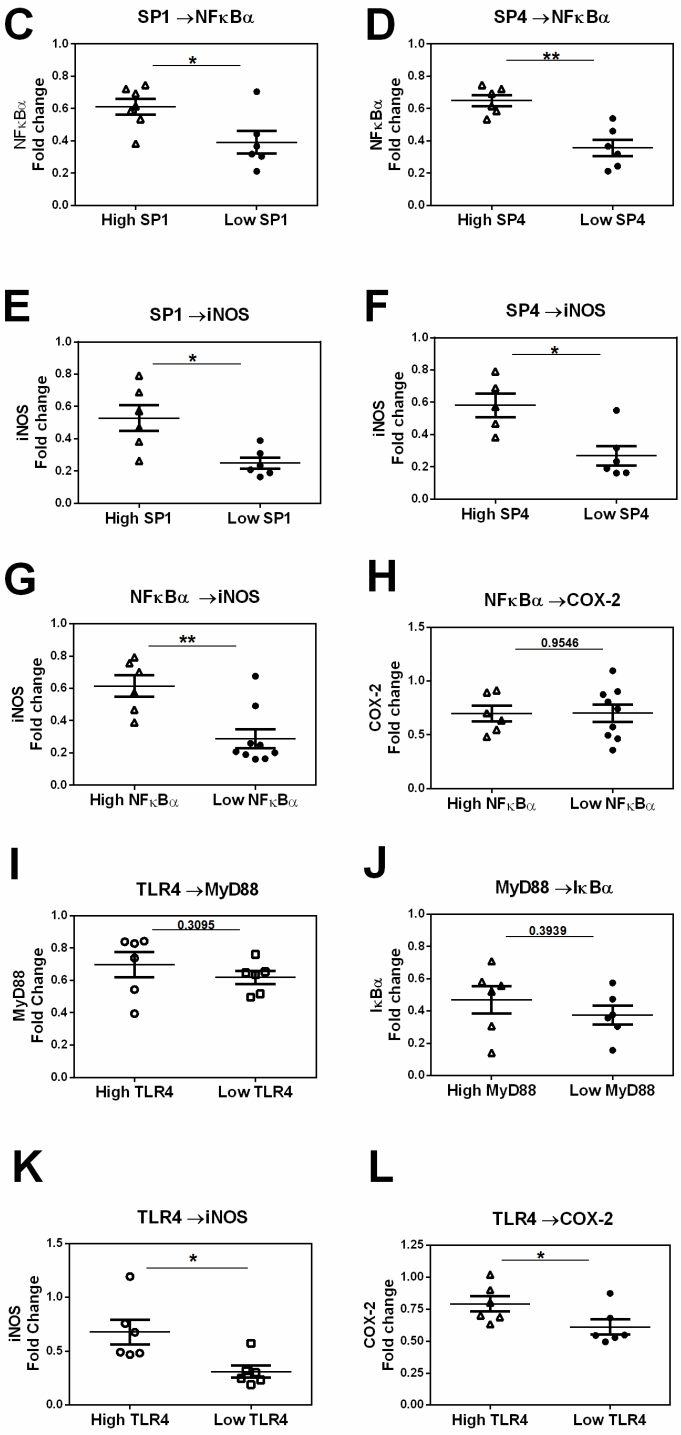


Table 1: Demographic, clinical and tissue-related features of cases.

	Chronic Schizophrenia	Non-psychiatric control	Statistic	p value
Prefrontal cortex	(n=15)	(n=14)		
Gender	Male- 100% (n=15)	Male- 100% (n=14)	N/A	N/A
Age at death	74 ± 9 years	74 ± 9 years	97.5[a]	0.760
PMD	4.9 ± 2.2 hours	5.7 ± 1.7 hours	1.08; 27[b]	0.290
pH	6.6 ± 0.4	6.8 ± 0.4	74.00[a]	0.173
Age of onset of illness	22 ± 7 years	N/A	N/A	N/A
Duration of illness	52 ± 10 years	N/A	N/A	N/A
D-A interval[c]	19 ± 14	N/A	N/A	N/A
Daily AP dose[d]	589 ± 491 mg/day	N/A	N/A	N/A
Clinical Scales		N/A	N/A	N/A
PANSS Positive	24.4 ± 7.7			
PANSS Negative	31.9 ± 10.6			
PANSS General	51.7 ± 15.3			
CGI-SCH Positive	4.3 ± 1.5			
CGI-SCH Negative	5.5 ± 1.1			
Cerebellum	(n=16)	(n=13)		
Gender	Male- 100% (n=16)	Male- 100% (n=13)	N/A	N/A
Age at death	75 ± 10 years	73 ± 8 years	82.50[a]	0.356
PMD	4.7 ± 2.4 hours	5.8 ± 1.7 hours	1.50; 27[b]	0.147
pH	6.6 ± 0.3	6.8 ± 0.6	1.33; 27[b]	0.193
Age of onset of illness	22 ± 7 years	N/A	N/A	N/A
Duration of illness	53 ± 10 years	N/A	N/A	N/A
D-A interval[c]	19 ± 13 months	N/A	N/A	N/A
Daily AP dose[d]	522 ± 497 mg/day	N/A	N/A	N/A
Clinical Scales		N/A	N/A	N/A
PANSS Positive	25.1 ± 8.0			
PANSS Negative	31.9 ± 10.2			
PANSS General	52.7 ± 15.3			
CGI-SCH Positive	4.5 ± 1.6			
CGI-SCH Negative	5.5 ± 1.0			

Mean ± standard deviation or relative frequency are shown for each variable; PMD, postmortem delay; D-A, Death to clinical assessment interval; AP, antipsychotic; PANSS, Positive and Negative Syndrome Scale; CGI-SCH, Clinical Global Impression-Schizophrenia Scale; N/A, not applicable. All deaths were due to natural causes. The same individuals were included for both brain areas with the following exceptions: one sample from the SZ group in the prefrontal cortex (PFC) and one sample from the control group in the cerebellum (CB) were not included in the molecular analysis due to the lack of sufficient tissue from this individual (PFC, SZ, n=15) or undetectable measures (CB, C, n=13). Comparison between groups (PFC: SZ, n=15, Control, n=14. CB: SZ, n=16; control, n=13) showed similar results in both areas.

[a]Mann-Whitney U is shown for non-parametric variables.

[b]T-statistic and degrees of freedom are shown for parametric variables.

[c]D-A Interval is defined as the number of months between clinical assessments and the time of death.

[d]Last chlorpromazine equivalent dose was calculated based on the electronic records of drug prescriptions of the patients.

Table 2: Association analysis of other variables in the study.

	Age	PMD	pH	CPZd (SZ only)	DI (SZ only)
SZ-C cohort					
Prefrontal cortex					
TLR4 protein	-0.061[1]	0.328[1]	-0.029[2]	-0.187[1]	-0.102[2]
MyD88 protein	-0.173[2]	**0.403[2a]**	-0.096[2]	-0.054[2]	-0.410[2]
IκBα protein	-0.360[1]	0.329[1]	-0.005[2]	0.082[1]	0.090[2]
COX2 protein	**-0.382[1a]**	-0.334[1]	0.323[2]	0.373[1]	-0.033[2]
MDA	0.158[2]	0.239[2]	**-0.387[2a]**	-0.252[2]	0.299[2]
Cerebellum					
TLR4 protein	0.280[1]	-0.238[1]	-0.157[1]	**-0.568[1a]**	0.163[2]
MyD88 protein	0.288[1]	-0.102[1]	0.127[1]	-0.260[1]	-0.053[2]
IκBα protein	-0.012[1]	0.115[1]	-0.265[1]	0.392[1]	0.322[2]
NFkB-activity	0.266[1]	0.039[1]	0.151[1]	-0.096[1]	0.118[2]
COX2 protein	-0.155[1]	-0.181[1]	0.095[1]	0.359[1]	-0.075[2]

SZ, schizophrenia; C, control; PMD, *post-mortem* delay; RIN, RNA Integrity Number; CPZd, chlorpromazine dose: last mean daily chlorpromazine equivalent dose; DI, duration of illness. Significant associations are indicated in bold.

[1]Pearson's r is shown for parametric variables.

[2]Spearman's r is shown for non-parametric variables.

[a]$p<0.05$.

Table 3: Associations between molecular measures and negative symptom scores.

Correlation	R Pearson	p value	FDR-adjusted p value	p value threshold
Prefrontal cortex				
NFκB activity-PANSS	-0.650	0.0119	**0.0952**	0.0125
NFκB protein-PANSS	-0.554	0.0322	0.1254	0.0250
NFκB activity-CGI-SCH	-0.620	0.0181	**0.0965**	0.0188
NFκB protein-CGI-SCH	-0.648	0.0090	**0.0952**	0.0063
IκBα protein-CGI-SCH[1]	0.536	0.0392	0.1581	0.0313
Cerebellum				
NFκB protein-CGI-SCH	-0.569	0.0269	0.3141	0.0063
IκBα protein-CGI-SCH	0.529	0.0425	0.3141	0.0125

FDR, False discovery rate; PANSS, Positive and Negative Syndrome Scale; CGI-SCH, Clinical Global Impression-Schizophrenia Scale. FDR-adjusted p values show p values adjusted for multiple comparisons and were calculated according to Benjamini and Hochberg method. The maximum acceptable FDR was set at 0.1 and the p value threshold yielded for each analysis is indicated. Significant p values after adjusting for the FDR are highlighted in bold.

[1]Spearman's correlation was applied and is shown in this case because the variable did not follow a normal distribution.

HIGHLIGHTS

- There are brain region-specific alterations in the TLR4 signaling pathway in chronic SZ.
- SP transcription factors modulates TLR4 signaling pathway in chronic SZ at different levels.
- NFκB activity in cerebellum is inversely related to negative symptoms in chronic SZ.