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SUPPLEMENTARY INFORMATION

METHODS AND MATERIALS

-Post-mortem human brain samples:

Table S1. Demographic characteristics, post-mortem delay (PMD), storage time, body mass index (BMI), RNA integrity number (RIN), cause of death/mechanism, brain pH and toxicological analysis of the schizophrenic subjects (Sch) and matched control subjects (C).

Antipsychotic-free schizophrenic subjects (n=17) and matched controls (n=17)										
Case/Control (Sch/C)	Gender (F/M)	Age (years)	PMD (hours)	Storage time (months)	BMI	RIN	Cause of death / Mechanism	Brain pH	Antipsychotics in blood	Other drugs in blood
Sch1	M	44	24	127	21	N/A	Suicide/Overdose	N/A		BZD, ETH (0.87 g/L), VEN, CMI
C1	M	44	21	56	31	8.5	Accident/Traffic	N/A		
Sch2	M	31	14	78	24	7.7	Suicide/Jumping	N/A		BZD
C2	M	32	28	79	25	8	Accident/Traffic	6.83		AMP, ETH (0.68 g/L)
Sch3	M	33	14	64	23	7.4	Suicide/Hanging	N/A		BZD
C3	M	33	4	51	31	9.1	Accident/Traffic	N/A		
Sch4	F	28	22	58	27	N/A	Suicide/Jumping	N/A		
C4	F	28	55	177	23	N/A	Accident/Traffic	N/A		
Sch5	M	50	3	72	28	N/A	Suicide/Drowned	7.09		BZD
C5	M	47	63	204	29	N/A	Accident/Traffic	N/A		ETH (1.16 g/L)
Sch6	F	75	48	44	30	N/A	Natural/CRF	N/A		PCT
C6	F	75	39	132	28	N/A	Accident/Traffic	N/A		
Sch7	M	49	41	195	32	N/A	Suicide/Hanging	N/A		ETH (0.49 g/L)
C7	M	45	30	178	23	6.3	Accident/Traffic	6.82		ETH (3.09 g/L)
Sch8	M	56	24	13	28	6.1	Natural/Heart attack	N/A		PCT
C8	M	58	20	23	25	6.2	Accident/Falling from a height	6.74		ETH (0.99 g/L)
Sch9	F	53	17	14	25	N/A	Natural/Haemorrhage	6.58		
C9	F	50	11	19	24	N/A	Natural/CRF	6.21		
Sch10	M	48	11	14	23	8	Suicide/Jumping	6.43		
C10	M	47	15	8	30	7.4	Accident/Traffic	6.15		ETH (1.55 g/L)
Sch11	M	35	15	4	19	8.5	Suicide/Hanging	6.2		
C11	M	37	14	187	23	N/A	Accident/Traffic	N/A		ETH (0.22 g/L)
Sch12	F	59	19	9	34	8.7	Natural/CRF	6.13		
C12	F	57	14	60	23	N/A	Natural/CRF	N/A		
Sch13	M	52	16	18	24	9.1	Suicide/Jumping	6.32		BZD
C13	M	51	18	3	30	N/A	Accident/Traffic	6.2		
Sch14	M	60	17	55	20	N/A	Natural/CRF	N/A		

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Antipsychotic-free schizophrenic subjects (n=17) and matched controls (n=17)										
Case/Control (Sch/C)	**Gender (F/M)**	**Age (years)**	**PMD (hours)**	**Storage time (months)**	**BMI**	**RIN**	**Cause of death / Mechanism**	**Brain pH**	**Antipsychotics in blood**	**Other drugs in blood**
C14	M	58	16	91	30	N/A	Accident/Traffic	N/A		ETH (0.77 g/L)
Sch15	M	45	13	58	25	9.3	Suicide/Jumping	7.19		BZD
C15	M	43	10	82	23	7.1	Accident/Traffic	N/A		ETH (2.5 g/L)
Sch16	M	49	19	11	30	N/A	Suicide/Jumping	6.40		
C16	M	51	13	10	29	8.1	Accident/Traffic	6.31		ETH (2.13 g/L)
Sch17	M	31	11	65	27	9.2	Suicide/Hanging	N/A		
C17	M	31	13	51	35	8.2	Accident/Traffic	N/A		ETH (0.96 g/L)
Group Sch	13 M/4 F	47±3	19±3	53±12	26±1	8.2±0.3		6.5±0.1		
Group C	13 M/4 F	46±3	23±4	83±17	27±1	7.7±0.2		6.5±0.1		
Sch18	M	30	18	82	25	8.2	Suicide/Jumping	6.98	OLZ	
C18	M	30	11	82	25	8	Accident/Electrocution	7.09		THC
Sch19	M	23	16	84	26	9.1	Suicide/Jumping	N/A	SLP	
C19	M	23	17	45	28	7.1	Accident/Electrocution	N/A		
Sch20	M	42	25	64	36	N/A	Natural/Heart attack	N/A	LVZ	
C20	M	42	27	127	24	N/A	Accident/Traffic	N/A		
Sch21	F	30	17	94	18	N/A	Suicide/Jumping	N/A	HLP	BZD
C21	F	32	18	73	21	N/A	Accident/Traffic	N/A		ETH (1.21 g/L)
Sch22	M	56	22	11	27	4	Natural/CRF	6.4	OLZ, CTP	
C22	M	54	24	3	27	N/A	Accident/Traffic	6.33		
Sch23	F	38	19	17	30	N/A	Suicide/Drowned	N/A	CTP, ZUC	BZD
C23	F	38	22	10	22	7.6	Accident/Traffic	6.44		
Sch24	M	35	11	22	30	8.6	Natural/CRF	6.01	CLZ	BZD
C24	M	36	18	92	30	9.2	Accident/Falling from a height	N/A		COC, ETH (1.69 g/L)
Sch25	M	42	14	22	23	N/A	Suicide/Overdose	6.2	RIS	BZD
C25	M	41	14	13	24	N/A	Natural/Heart attack	6.81		
Sch26	M	37	18	27	23	7.1	Suicide/Burnt	7.04	OLZ	BZD, ETH (0.9 g/L)
C26	M	36	23	3	33	8.2	Accident/Crushing	6.42		
Sch27	F	48	17	72	29	N/A	Suicide/Jumping	N/A	OLZ, CTP	
C27	F	45	12	20	31	N/A	Natural/CRF	6.37		
Sch28	M	62	10	96	26	N/A	Natural/CRF	N/A	THI	
C28	M	61	23	92	27	8.0	Accident/Traffic	7.0		ETH (0.72 g/L)
Sch29	M	72	14	71	27	N/A	Natural/CRF	N/A	PER	BZD
C29	M	71	21	79	28	N/A	Natural/CRF	N/A		
Sch30	F	69	11	62	28	N/A	Natural/CRF	N/A	THI	BZD

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Case/Control (Sch/C)	Gender (F/M)	Age (years)	PMD (hours)	Storage time (months)	BMI	RIN	Cause of death / Mechanism	Brain pH	Antipsychotics in blood	Other drugs in blood
Antipsychotic-free schizophrenic subjects (n=17) and matched controls (n=17)										
C30	F	70	7	74	34	N/A	Accident/Traffic	N/A		
Group Sch	9 M/4 F	45±4	16±1	56±9	27±1	7.4±0.9		6.5±0.2		
Group C	9 M/4 F	44±4	18±2	55±11	27±1	8.0±0.3		6.6±0.1		
AP-free and AP-treated schizophrenic subjects (n=30) and matched controls (n=30)										
Group Sch	22 M/8 F	46±2	18±2	54±8	26±1	8.1±0.2		6.5±0.1		
Group C	22 M/8 F	45±2	21±2	71±11	27±1	7.8±0.2		6.5±0.1		

Group values are means ± SEM. F (female), M (male), CRF (cardio-respiratory failure), N/A (not available). Drugs in blood are coded as: AMP (amphetamine), BZD (non-specified benzodiazepines or metabolites), COC (cocaine), CLZ (clozapine), CMI (clomipramine), CTP (clotiapine), ETH (ethanol), HLP (haloperidol), LVZ (levomepromazine), OLZ (olanzapine), PCT (paracetamol), PER (pericyazine), RIS (risperidone), SLP (sulpiride), THC (tetrahidrocannabinol), THI (thioridazine), VEN (venlafaxine), ZUC (zuclopenthixol).

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-Preparation of nuclear extracts:

Tissues (brain prefrontal cortex) were 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 reach a concentration of 0.5%. The tubes were gently vortexed for 15 sec, 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 steps in the fractionation were carried out at 4ºC.

-Western blot analysis:

After adjusting the protein levels in the resultant supernatants, homogenates were mixed with Laemmli sample buffer (BioRad, Hercules, CA, USA) and 10 µL (1 mg/mL) was 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 twice. 2 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/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 at 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 at the C-terminus of COX-2 of mouse

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origin at a dilution of 1:750 in 5% BSA in TBS-Tween) (sc-1747); NF-κB p65 subunit (rabbit polyclonal antibody raised against an epitope mapping within the C-terminus of NF-κB p65 of human origin at a dilution of 1:500 in BSA 2%) (sc-372); IκBα (rabbit polyclonal antibody raised against a peptide mapping at the C-terminus of IκBα of human origin at a dilution of 1:1000 in BSWA 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); PPARγ (rabbit polyclonal antibody raised against an epitope corresponding to amino acids 8-106 mapping at the N-terminus of PPARγ1 of human origin) (H-100). 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 analysis, the housekeeping gene 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 case of the NF-κB p65 subunit in which the loading control was the nuclear factor SP1 (rabbit polyclonal antibody at a dilution of 1:2000 (sc-59; Santa Cruz Biotechnology) (blots shown in the respective figures).

-Real-time polymerase chain reaction analysis:

The primers used were as detailed here. The MyD88 forward primer (5'-3'), CTGGCTGTTGCTGGACTACA, and reverse primer (5'-3'), TGAGAGGTGGACCCATTAGG. The IL-1β forward

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primer (5'-3'), GGCTGCTCTGGGATTCTCTT, and reverse primer (5'-3'), CTGGCGAGCTCAGGTACTTC. The IL-6 forward primer (5'-3'), ATGCAATAACCACCCCTGAC, and reverse primer (5'-3'), GAGGTGCCCATGCTACATTT.

-Lipid peroxidation:

Lipid peroxidation was measured using a modification of the method of Das & Ratty (1987), whereby the 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 90°C and kept at that temperature for 15 min, then centrifuged at 12000g 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.

-Nitrite (NO_2^-) levels:

As the stable metabolites of the free radical nitric oxide ($NO^\cdot$), NO_2^- were measured using the Griess method (Green et al., 1982). In an acidic solution with 1% sulphanilamide and 0.1% NEDA, nitrites convert into a pink compound that is photometrically calculated at 540 nm in a microplate reader (Synergy 2; BioTek, USA).

GENETIC ASSOCIATION STUDY

-Statistics:

SNP interaction analysis: seven correlated SNPs (r^2>0.8) were excluded from the analysis to remove noisy SNPs from the pool of possible candidates. We constructed all possible SNP combinations by testing all possible two- to five-locus interactions. Cross-validations were repeated 100 times and exhaustive searches were selected. As outcome parameters, we considered cross-validation consistency (which

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measures the consistency of the identification of the variations selected on the basis of the best model applied to the computation), testing balanced accuracy (which measures the degree to which an interaction accurately predicts case-control status) and statistical significance (the p-value of the best model). Then, we created a new multilocus attribute with the best model obtained. This is the model with the best outcome parameters as described earlier. The new attribute was constructed and reanalysed in order to calculate the statistics for the whole dataset, obtaining the odds ratio (OR) and its confidence interval, p-value, sensitivity (which measures the capacity to correctly predict patients), specificity (which measures the capacity to correctly predict controls), accuracy (which measures the capacity to correctly predict patients and controls) and precision (which measures the correctly predicted patients).

RESULTS

Table S2. Correlative analysis between major significant findings

	TLR-4 prot		**MyD88 prot**		**NF-κB prot**		**MDA**	
	R value	P value	R value	P value	R value	P value	R value	P value
TLR-4 prot			0.003	0.9893	0.039	0.8359	0.037	0.8456
MyD88 prot	0.325	0.0792			0.286	0.1257	0.005	0.9809
NF-κB prot	0.133	0.4836	**0.601**	**0.0005**			-0.227	0.2276
MDA	-0.069	0.7165	0.348	0.0597	0.218	0.2481		

TLR-4: toll-like receptor 4; MyD88: myeloid differentiation factor 88; NF-κB: nuclear factor κB; MDA: malondialdehyde; prot: protein. R (correlation coefficient) and p values above black boxes correspond to control subjects. R and p values below black boxes correspond to SZ subjects.

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FIGURE S1

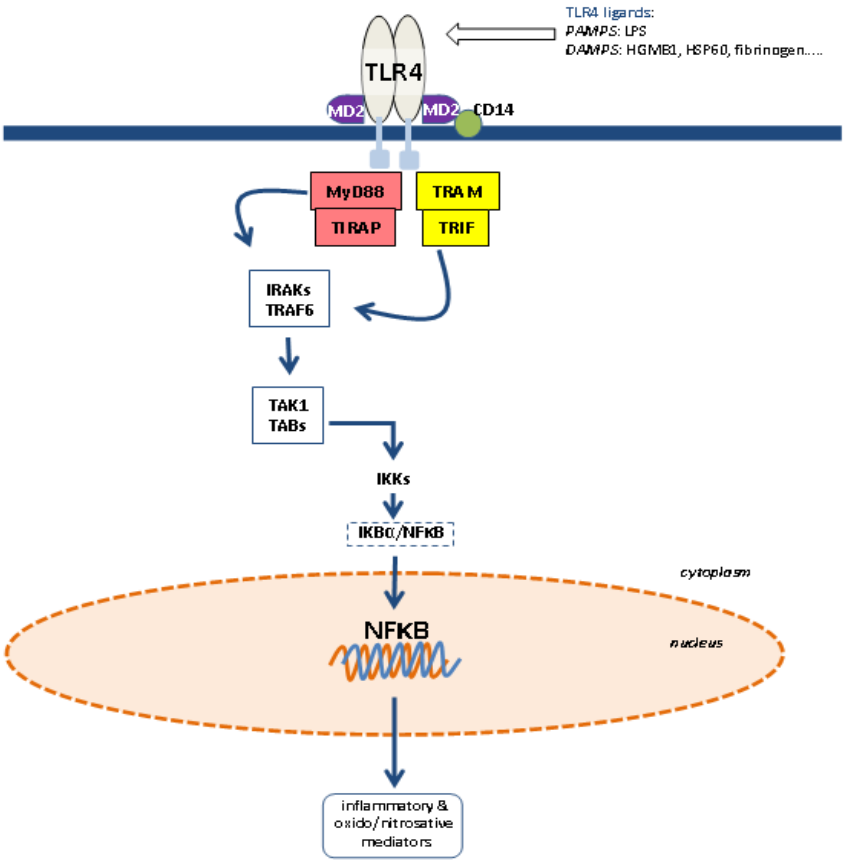

Figure S1. MyD88-dependent elements of the TLR-4 pathway
After ligand binding and the subsequent receptor homodimerization/heterodimerization, MyD88 or TRIF is recruited through the bridging adaptors TIRAP and TRAM, respectively. A frequent inducer of downstream signalling is MyD88 association with IRAK (IL-1 receptor-associated kinase), TRAF (TNF receptor-associated factor), TAK1 (transforming growth factor β (TGF-β)-activated kinase 1) and its binding protein (TAB), followed by subsequent activation of IKK kinases, which phosphorylates IκBα, thereby facilitating the nuclear translocation of NF-κB, where it activates the transcription of inflammatory cytokines and other inflammatory/oxidative/nitrosative stress mediators.

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SUPPLEMENTARY REFERENCES

-Das NP, Ratty AK. Studies on the effects of the narcotic alkaloids, cocaine, morphine and codeine on nonenzymatic lipid peroxidation in rat brain mitochondria. *Biochem Med Metab Biol* 1987*;* 37: 256–264.

-Schreiber E, Matthias P, Müller MM et al. Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acid Res* 1989; 17: 6419.

SUPPLEMENTARY REFERENCES TABLE 1

62. Chien JW, Boeckh MJ, Hansen JA, et al. Lipopolysaccharide binding protein promoter variants influence the risk for Gram-negative bacteremia and mortality after allogeneic hematopoietic cell transplantation. Blood 2008; 111(4):2462-9.

63. Inoue Y, Shimojo N, Suzuki Y, et al. CD14 -550 C/T, which is related to the serum level of soluble CD14, is associated with the development of respiratory syncytial virus bronchiolitis in the Japanese population. J Infect Dis 2007; 195(11):1618-24.

64. Mohamed JA, DuPont HL, Flores J, et al. Single nucleotide polymorphisms in the promoter of the gene encoding the lipopolysaccharide receptor CD14 are associated with bacterial diarrhea in US and Canadian travelers to Mexico. Clin Infect Dis. 2011; 52(11):1332-41.

65. Sato K, Yoshimura A, Kaneko T, et al. A single nucleotide polymorphism in 3'-untranslated region contributes to the regulation of Toll-like receptor 4 translation. J Biol Chem 2012; 287(30):25163-72.

66. Daniil Z, Mollaki V, Malli F, et al. Polymorphisms and haplotypes in MyD88 are associated with the development of sarcoidosis: a candidate-gene association study. Mol Biol Rep 2013; 40(7):4281-6.

67. Yin J, Yao CL, Liu CL, Song ZJ, Tong CY, Huang PZ. Association of genetic variants in the IRAK-4 gene with susceptibility to severe sepsis. World J Emerg Med 2012; 3(2):123-7.

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DOI: 10.1503/jpn.150195

Online appendices are unedited and posted as supplied by the authors.

68. Zhang H, Pu J, Wang X, et al. IRAK1 rs3027898 C/A polymorphism is associated with risk of rheumatoid arthritis. Rheumatol Int 2013; 33(2):369-75.

69. Karban AS, Okazaki T, Panhuysen CI, et al. Functional annotation of a novel NFKB1 promoter polymorphism that increases risk for ulcerative colitis. Hum Mol Genet 2004; 13(1):35-45.

70. Hayashi R, Tahara T, Yamaaki T, et al. -449 C>G polymorphism of NFKB1 gene, coding nuclear factor-kappa-B, is associated with the susceptibility to ulcerative colitis. World J Gastroenterol 2012; 18(47):6981-6.

71. Liou YJ, Wang HH, Lee MT, et al. Genome-wide association study of treatment refractory schizophrenia in Han Chinese. PLoS One 2012; 7(3):e33598.

72. Gao SP, Liang S, Pan M, et al. Interleukin-6 genotypes and serum levels in Chinese Hui population. Interleukin-6 genotypes and serum levels in Chinese Hui population. Int J Clin Exp Med 2014; 7(9):2851-7.

73. Martínez-Carrillo DN, Garza-González E, Betancourt-Linares R, et al. Association of IL1B -511C/-31T haplotype and Helicobacter pylori vacA genotypes with gastric ulcer and chronic gastritis. BMC Gastroenterol 2010; 10:126.